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(54) Title: PRODUCTION OF ATTENUATED PARAINFLUENZA VIRUS VACCINES FROM CLONED NUCLEOTIDE SEQUENCES

(57) Abstract

Isolated polynucleotide molecules provide recombinant PIV genomes and antigenomes for production of recombinant PIV vaccines. The recombinant genome or antigenome can be expressed with a nucleoprotein (N), phosphoprotein (P), and a large (L) polymerase protein to produce isolated infectious PIV particles. The recombinant PIV genome and antigenome can be modified to produce desired changes, for example to incorporate attenuating mutations from biologically derived PIV mutants or to create chimeric PIV clones, to generate attenuated, immunogenic viruses for vaccine use.

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PRODUCTION OF ATTENUATED PARAINFLUENZA VIRUS VACCINES FROM CLONED NUCLEOTIDE SEQUENCES

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Related Applications

The present application is a continuation-in-part application of, and claims the benefit under Title 35 of U.S.

15   Provisional Application No. 60/047,575, filed May 23, 1997, and U.S. Provisional Application No. 60/059,385, filed September 19, 1997, each of which is incorporated herein by reference.

20   Background of the Invention

Human parainfluenza viruses (HPIV), HPIV1, HPIV2, and HPIV3 are significant causes of bronchiolitis, croup and pneumonia in infants and children. Karron et al., J. Infect. Dis. 172: 1445-50 (1995); Collins et al. "Parainfluenza

25   Viruses", p. 1205-1243. In B. N. Fields et al., eds., Fields Virology, 3rd ed, vol. 1. Lippincott-Raven Publ., Philadelphia (1996); Murphy et al., Virus Res. 11:1-15 (1988). Infections by these viruses result in substantial morbidity in children less than 3 years of age, and are responsible for

30   approximately 20% of hospitalizations among young infants and children for respiratory tract infections.

Despite considerable efforts to develop effective vaccine therapies against HPIV, no approved vaccine agents have yet been achieved for any HPIV strain, nor for

35   ameliorating HPIV related illnesses. To date, only two live attenuated PIV vaccine candidates have received particular attention. One of these candidates is a bovine PIV (BPIV) strain that is antigenically related to HPIV3, and which has been shown to protect animals against HPIV3. BPIV3 is

40   attenuated, genetically stable and immunogenic in human infants and children (Karron et al., J. Inf. Dis. 171:1107-14

(1995a); Karron et al., _J. Inf. Dis._ 172:1445-1450, (1995b)).
A second PIV3 vaccine candidate, JS _cp_45 is a cold-adapted
mutant of the JS wildtype (wt) strain of HPIV3 (Karron et al.,
(1995b), _supra_; Belshe et al., _J. Med. Virol._ 10:235-42

5   (1982)). This live, attenuated, cold-passaged (_cp_) PIV3
vaccine candidate exhibits temperature-sensitive (_ts_),
cold-adaptation (_ca_), and attenuation (_att_) phenotypes which
are stable after viral replication _in vivo_. The _cp_45 virus is
protective against human PIV3 challenge in experimental

10   animals and is attenuated, genetically stable, and immunogenic
in seronegative human infants and children (Hall et al.,
Virus Res. 22:173-184 (1992); Karron et al., (1995b), _supra_.

         HPIV3 is a member of the Paramyxovirus genus of the
Paramyxovirus family, order Mononegavirales. Its genome is a

15   single strand of negative-sense RNA 15462 nucleotides (nt) in
length (Galinski et al., _Virology_ 165: 499-510, (1988); Stokes
et al., _Virus Res._ 25:91-103 (1992)) and encodes at least
eight proteins (Collins et al., _supra_, (1996); Galinski,
supra, (1991); Spriggs and Collins, _J. Gen. Virol._ 67: 2705-

20   2719, (1986)). Three of these proteins are associated with
the RNA genome to form the nucleocapsid; namely the
nucleocapsid protein N, phosphoprotein P, and large polymerase
subunit L. Three additional proteins are associated with the
envelope, namely the matrix protein M, taught to mediate viral

25   attachment and release, the hemagglutinin-neuraminidase
protein HN, and the fusion protein F. Two other proteins, HN
and F, represent the neutralizing and protective antigens of
PIVs (Collins et al. In _Fields Virology_, 3rd ed., 1:1205-43
(1996)). Significant sequence divergence in these two

30   protective antigens among different PIVs is the basis for the
type specificity of protective immunity against these
pathogens (_id._).

         Another protein of PIV, the C protein, is encoded by
an overlapping open reading frame (ORF) of the P protein mRNA

35   (Spriggs and Collins, 1986), and the D protein is generated by
RNA editing of the P cistron (Galinski et al. _Virology_
186:543-50 (1992)). The P mRNA also contains an internal ORF
which has the potential to encode a cystein-rich domain called

V.   The V ORF is also found in other paramyxoviruses and
typically is accessed by RNA editing, but this is not the case
with PIV.   Presently, it is not known whether the PIV V ORF is
expressed.

5   The viral genome of PIV also contains extragenic
leader and trailer regions, possessing promoters required for
viral replication and transcription.   Thus, the PIV genetic
map is represented as 3' leader-N-P/C/D-M-F-HN-L-trailer.
Transcription initiates at the 3' end and proceeds by a

10   sequential stop-start mechanism that is guided by short
conserved motifs found at the gene boundaries.   The upstream
end of each gene contains a gene-start (GS) signal, which
directs initiation of its respective mRNA.   The downstream
terminus of each gene contains a gene-end (GE) motif which

15   directs polyadenylation and termination.
           Identification of attenuating mutations in cp45 and
other PIV3 vaccine candidates is of interest for a variety of
reasons.   In particular, it will be useful to understand the
genetic basis of attenuation and to characterize the molecular

20   virology and pathogenesis of attenuated HPIV3 strains to
provide clinically acceptable vaccines for use against these
and other paramyxoviruses, especially HPIV1 and HPIV2 which
together account for an additional 7% of pediatric hospital
admissions.   To achieve these and related goals, a method for

25   producing virus with a wt phenotype from cDNA is needed to
determine which mutation(s) in the cp45 virus specify the ts,
ca and att phenotypes and which gene(s) of the BPIV3 specify
the attenuation phenotype.
           The complete nucleotide sequences of the prototype

30   PIV3 strain, and of the JS wt HPIV3 and cp45 strains have been
determined (Stokes et al., supra., (1992); Stokes et al.,
Virus Res. 30: 43-52 (1993)).   From these studies, the cp45
strain was shown to possess at least seventeen nucleotide
substitutions compared to the parental JS wt HPIV3 strain,

35   eight of which are correlated with changes to viral proteins.
However, it has not been previously shown which of these
identified mutations specify desired, e.g., ts, ca, and att,
phenotypes.   Recently, growth of cp45 at nonpermissive

4

temperatures was reported to be complemented by coexpression of a cDNA clone of the L gene of the 47885 wt PIV3 strain (Ray et al., _J. Virol._ 70:580-584 (1996)), suggesting that the L gene may contain one or more mutations which contribute to the

5   _ts_ phenotype of _cp_45. However, the results of this study are complicated by the fact that the 47885 strain is not isogenic with the JS parent of _cp_45 (for example, the two viruses are 4% different at the nucleotide level, and the L proteins differ at 41 amino acid positions (Stokes et al., _supra_,

10   (1992); published erratum appears in Virus Res. 27:96 (1993); _Virus Res._ 25:91-103. Also, this method of complementation does not provide a clear measurement of the relative contribution of the L gene mutation(s) to the overall _ts_ phenotype of _cp_45.

15   Rescue and analysis of attenuating mutations in PIV3 and other RNA viruses require effective methods to manipulate cDNAs for the particular viruses of interest. Despite previous advancements identifying cDNAs for PIV, manipulation of the genomic RNA of this and other negative-sense RNA

20   viruses has proven difficult. One major obstacle in this regard is that the naked genomic RNA of these viruses is noninfectious.

          Successful methods for direct genetic manipulation of non-segmented negative strand RNA viruses have only

25   recently begun to be developed (for reviews, see Conzelmann, _J. Gen. Virol._ 77:381-89 (1996); Palese et al., _Proc. Natl._ _Acad. Sci. U.S.A._ 93:11354-58, (1996)). Functional nucleocapsids have been successfully generated from the intracellular coexpression of separately transfected plasmids

30   bearing the T7 RNA polymerase promoter and encoding either genomic or antigenomic RNA and the N, P, and L proteins. The intracellular cDNA expression is driven by T7 RNA polymerase which is produced by co-infecting with a vaccinia recombinant virus. This approach was first used to determine the

35   transcription and replication requirements of cDNA-encoded minireplicons. Some success has been achieved in the application of these general methods to rescue infectious rabies virus, vesicular stomatitis virus (VSV), measles virus,

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and Sendai virus from cDNA-encoded antigenomic RNA in the
presence of the nucleocapsid N, phosphoprotein P, and large
polymerase subunit L (Garcin et al., EMBO J. 14:6087-6094
(1995); Lawson et al., Proc. Natl. Acad. Sci. U.S.A.
5   92:4477-81 (1995); Radecke et al., EMBO J. 14:5773-5784
(1995); Schnell et al., EMBO J. 13:4195-203 (1994); Whelan et
al., Proc. Natl. Acad. Sci. U.S.A. 92:8388-92 (1995)).
Respiratory synctial virus (RSV) has also been recovered from
cDNA encoded antigenome but this required the transfection of
10   an additional plasmid encoding the M2 ORF 1 transcription
elongation factor (Collins et al., 1995).

Rescue of infectious PIV virus and other
Mononegavirales members is complicated by virtue of their non-
segmented negative-strand RNA genome. The genomic
15   ribonucleoprotein complexes (RNPs) of segmented genome
viruses, such as influenza, are generally small in size and
loosely structured, and can be assembled in vitro from RNA and
required viral proteins. However, PIV and other
Mononegavirales members feature much larger and more tightly
20   structured RNPs, which tend to be refractory to functional
association in vitro. Furthermore, the coding potential of
HPIV3 P mRNA is complicated by cotranscriptional "RNA editing"
(Galinski et al., Virology 186: 543-50 (1992)). The resultant
shifts in reading frame can access internal ORFs which are
25   expressed as chimeras fused to the N-terminal part of P. In
addition, HPIV3 appears to differ from most other
paramyxoviruses which express a chimeric V protein, as noted
above. The corresponding set of proteins from HPIV3 editing
has not yet been identified, and the internal V ORF of HPIV3
30   is separated from the editing site by numerous translational
stop codons (Galinski et al. (1992, supra). Yet another
complicating factor is that editing by BPIV3 and HPIV3
produces a novel chimeric protein D, in which the upstream
half of P is fused to a domain encoded by a second internal
35   ORF (Pelet et al., EMBO J. 10: 443-448 (1991); Galinski et
al., supra, (1992)). The D protein does not have a
counterpart in other paramyxoviruses.

6

In view of the foregoing, an urgent need exists in the art for tools and methods to engineer safe and effective vaccines to alleviate the serious health problems attributable to PIV, particularly illnesses among infants and children attributable to HPIV3. Quite surprisingly, the present invention satisfies these and other related needs.

Summary Of The Invention

The present invention provides novel tools and methods for introducing defined, predetermined structural and phenotypic changes into infectious PIV. In one embodiment of the invention, an isolated polynucleotide molecule is provided which comprises an operably linked transcriptional promoter, a polynucleotide sequence encoding a PIV genome or antigenome, and a transcriptional terminator.

The PIV genome or antigenome can be a human or nonhuman PIV sequence, or a recombinantly modified version thereof. In one embodiment, the polynucleotide sequence encodes a chimeric genome or antigenome comprising a human PIV sequence recombinantly joined with a nonhuman PIV sequence, such as a gene or gene segment from bovine PIV (BPIV). In additional examples, the polynucleotide encodes a chimera of sequences from a nonhuman PIV and at least one other PIV of human or nonhuman origin.

In other embodiments, the invention provides an isolated infectious PIV particle comprising a recombinant PIV (rPIV) genome or antigenome. The isolated infectious PIV particle can be a viral or subviral particle. As used herein, subviral particle refers to any infectious PIV particle which lacks a structural element, eg., a gene segment, gene, protein, or protein functional domain, which is present in a complete virus (eg., an assembled virion including a complete genome or antigenome, nucleocapsid and envelope). Thus, one example of a subviral particle of the invention is an infectious nucleocapsid containing a genome or antigenome, and the products of N, P, and L genes. Other subviral particles are produced by partial or complete deletions or substitutions

of non-essential genes and/or their products (eg., F, HN, M,
or C), among other non-essential structural elements.

The isolated infectious PIV particle is preferably a
human PIV, more preferably human PIV3 (HPIV3). The invention
also provides isolated, infectious particles from bovine or
murine PIV (BPIV or MPIV), as well as particles comprising
chimeric sequences from two or more different PIV genomes, for
example particles incorporating polynucleotide sequences from
HPIV3 and HPIV1, from HPIV3 and HPIV2 sequences, or comprised
of HPIV3 and BPIV sequences.

In related aspects of the invention, isolated,
infectious PIV particles are provided which incorporate
nucleotide sequences from HPIV3 joined to at least one
sequence from a heterologous PIV, such as HPIV1, HPIV2, BPIV
or MPIV. For example, entire genes of HPIV3 may be replaced
by counterpart genes from other forms of PIV, such as the HN
and/or F glycoprotein genes of PIV1 or PIV2. Alternatively, a
selected gene segment, for example a cytoplasmic tail,
transmembrane domain or ectodomain of HN or F of HPIV1 or
HPIV2, can be substituted for a corresponding gene segment in
a counterpart HPIV3 gene to yield constructs encoding chimeric
proteins, e.g. fusion proteins having a cytoplasmic tail
and/or transmembrane domain of PIV3 fused to an ectodomain of
PIV1 or PIV2. Alternatively, genes or gene segments from one
PIV can be added (i.e., without substitution) within a
heterologous PIV background to create novel immunogenic
properties within the resultant clone.

Other modifications can be produced by introducing
into a PIV genome or antigenome a nucleotide insertion,
rearrangement, deletion or substitution selected to encode a
desired phenotypic alteration, such as one that results in
attenuation, temperature-sensitivity, cold-adaptation, small
plaque size, host range restriction, improved growth in
vitro.or a change in an immunogenic epitope of PIV. In one
aspect of the invention, mutations occurring in biologically
derived, attenuated PIV are identified and introduced
individually or in combination into a full-length PIV clone.
Typically these mutations are single amino acid changes

8   -

displayed by biologically derived mutant viruses over a wild-
type PIV, for example changes exhibited by PIV mutants having
ts, ca or *att* phenotypes. These changes from biologically
derived mutant PIV are incorporated into a recombinant PIV
clone to specify desired characteristics in the resultant
virus. Exemplary mutations include amino acid changes which
specify an attenuated phenotype in the HPIV3 strain JS *cp*45.
Among these exemplary mutations are mutations occurring within
the PIV polymerase gene L specifying *ts, ca* or *att* phenotypes,
for example amino acid substitutions occurring at Tyr_{942},
Leu_{992}, and/or Thr_{1558} of the JS wild type PIV strain. In more
detailed aspects, attenuated PIV recombinants are described
wherein Tyr_{942} is replaced by His, Leu_{992} is replaced by Phe,
and/or Thr_{1558} is replaced by Ile.

        Also provided within the invention are recombinant
PIV having multiple, phenotype-specifying mutations introduced
in selected combinations into the genome or antigenome of an
infectious clone to yield desired characteristics including
attenuation, temperature sensitivity, cold-adaptation, small
plaque size, host range restriction, etc. For example, PIV
clones are provided which incorporate at least two separate
mutations adopted from a biologically derived PIV mutant,
e.g., two *ts* mutations from HPIV3 JS *cp*45. Multiply
attenuated viruses are thus obtained by selecting mutations
from a "menu" of identified lesions and introducing these
mutations in various combinations to calibrate a vaccine virus
to selected levels of attenuation, immunogenicity and
stability.

        In additional embodiments, the invention provides
for supplementation of one or more mutations adopted from
biologically derived PIV, e.g., *ts, ca* or *att* mutations, with
additional types of mutations involving the same or different
genes. Target genes for mutation in this context include the
nucleocapsid protein N, phosphoprotein P, large polymerase
subunit L, matrix protein M, hemagglutinin-neuraminidase
protein HN, fusion protein F and the C, D and V ORF products.
In preferred aspects, attenuating mutations adopted from
biologically derived PIV are incorporated within a chimeric

PIV recombinant, e.g., a PIV recombinant having nucleotide
sequences from both HPIV3 and HPIV1, or from both HPIV and
BPIV viruses.

In other embodiments, the invention provides methods
5   for producing an infectious PIV particle, e.g, a viral or
subviral particle, from one or more isolated polynucleotide
molecules encoding a PIV genome or antigenome (see also
copending United States provisional patent application No.
60/047575, filed May 23 1997, incorporated herein by reference
10   in its entirety). To produce an infectious PIV particle
according to these methods, an expression vector comprising an
isolated polynucleotide molecule encoding a PIV genome or
antigenome is coexpressed in a cell or cell-free system with
an expression vector comprising one or more isolated
15   polynucleotide molecules encoding N, P, and L proteins of a
PIV, whereby an infectious PIV particle is produced.

The PIV genome or antigenome and the N, P, and L
proteins may be coexpressed by a single expression vector, or
by separate expression vectors. In alternate embodiments, the
20   N, P, and L proteins are each encoded on separate expression
vectors.

Within the aforementioned methods, the
polynucleotide molecule encoding the PIV genome or antigenome
may correspond to a genomic or antigenomic sequence of human,
25   bovine or murine PIV. Alternatively, the PIV encoding
polynucleotide may be a chimera of a human PIV genomic or
antigenomic sequence and at least one non-human PIV genomic or
antigenomic sequence. In additional methods for producing
infectious PIV, the polynucleotide encoding the PIV genome or
30   antigenome is a chimera of two or more human PIV genomes, for
example a polynucleotide containing sequences from HPIV3
joined to sequences from one or more related forms of human
PIV, such as human PIV1 or human PIV2. Individual genes of
human PIV3 may be substituted by counterpart genes from
35   heterologous PIV, for example the HN and F glycoprotein genes
of PIV1 or PIV2, to yield a modified genome or antigenome
encoding a chimeric PIV. Alternatively, a selected,
heterologous gene segment, such as a cytoplasmic tail,

transmembrane domain or ectodomain of HN or F of HPIV1 or
HPIV2, can be substituted for a counterpart gene segment in a
different PIV type or different gene, e.g., HN or F of HPIV3,
to yield constructs encoding chimeric proteins, e.g. fusion
proteins having a cytoplasmic tail and/or transmembrane domain
of PIV3 fused to an ectodomain of PIV1 or PIV2.

In yet additional methods for producing infectious
PIV, the PIV genome or antigenome is modified to yield a
chimera of a human PIV genomic or antigenomic sequence and at
least one non-human PIV sequence, for example a polynucleotide
containing sequences from both human and bovine PIV.

In other methods for producing infectious PIV, the
PIV genome or antigenome is modified by a nucleotide
insertion, rearrangement, deletion or substitution selected to
encode a desirable phenotypic alteration, such as one that
results in attenuation, temperature-sensitivity, cold-
adaptation, small plaque size, host range restriction, or a
change in an immunogenic epitope of PIV. Alternatively, the
polynucleotide molecule encoding the PIV genome or antigenome
can be modified to encode non-PIV molecules, e.g., a cytokine,
a T-helper epitope, a restriction site marker, or a protein of
a different microbial pathogen (e.g., virus, bacterium or
fungus) capable of eliciting a protective immune response in
the intended host. In one embodiment, the PIV genome or
antigenome is modified to encode protein from a human RSV or
from measles virus.

In other embodiments of the invention a cell or
cell-free expression system (e.g., a cell-free lysate) is
provided which incorporates an expression vector comprising an
isolated polynucleotide molecule encoding a PIV genome or
antigenome, and an expression vector comprising one or more
isolated polynucleotide molecules encoding N, P, and L
proteins of a PIV. Upon expression, the genome or antigenome
and N, P, and L proteins combine to produce an infectious PIV
particle, such as a viral or subviral particle. The isolated
polynucleotide molecules encoding the PIV genome or antigenome
and the one or more isolated polynucleotide molecules encoding
N, P, and L proteins of PIV can be expressed by a single

vector, or the genome and one or more of the N, P, and L
proteins can be incorporated into two or more separate
vectors.

5   Brief Description of the Drawings
         Figure 1 illustrates construction of the plasmid
p218(131), encoding a complete copy of HPIV3 genomic RNA. The
diagram of p218(A*A'CC'D*E) (top left) illustrates the
location of the T7 terminator and delta ribozyme adjacent to
10 nucleotide 1 of the HPIV3 genome. The top right diagram of
p(E*FF'GHIJKL*) illustrates the locations of the 7 sequencing
markers (asterisk), see Fig. 2 below, and the T7 promoter
adjacent to nucleotide 15462 of HPIV3. The 15 overlapping
clones used to construct the full length genomic cDNA are
15 indicated by letters on the outside of the two plasmids. The
unique restriction sites *NgoMI* and *XhoI* were used to clone
p218(131) which encodes the complete negative-sense genomic
RNA of HPIV3 illustrated at the bottom. The location of each
sequencing marker in p218(131) differentiating recombinant JS
20 from JS wt is indicated by an asterisk.
         Figure 2 depicts sequence markers in cDNA encoded
genomic RNA of the HN (1) and L(2) genes of HPIV3. Sequences
are negative sense, mutations are boxed.
         1) A non-coding nt substitution at 7903 removes a
25 *Hga* I site from JS wt. Nucleotide substitutions in JS cDNA at
7913 and 7915 create a *Sca* I site and change amino acid 370
from proline to threonine, ablating the antibody epitope
recognized by mAb 170/7 and 423/6. Also, the HN gene was
found to contain an additional point mutation at nt 7593 which
30 had not been recognized previously. This results in a
threonine to isoleucine change at amino acid position 263 in
the HN protein.
         2)   The three incidental non-coding mutations in the
L gene of JS cDNA that occurred during plasmid construction or
35 assembly are boxed.
         Figure 3 includes a diagram (not to scale) of the
PIV3-CAT cDNA (top, drawn as a circular plasmid) and the

12   —

encoded minigenome RNA (bottom, drawn as a single strand of
negative-sense RNA, 3´ to 5´).

Plasmid: The T7 promoter is shown as a black arrow
pointing in the direction of transcription. Positions are
5   shown for the delta ribozyme, the T7 transcriptional
terminator, the inserted vaccinia virus transcriptional
terminators (T$_7$CT and T$_5$AT), restriction enzyme sites, and the
coding sequence for the various domains of the encoded
minigenome.

10   Minigenome RNA: The 5' (right hand) end of the
minigenome is defined by the promoter for T7 RNA polymerase,
which contributes an extension of two nonviral G residues.
These are not included in length calculations. The 3' end is
defined by the ribozyme and is free of any heterologous
15   nucleotides. PIV3-specific regions are shown as open boxes.
The CAT ORF is shaded and the negative-sense complements of
the translational initiation (UAC) and termination (AUU)
codons are shown. Nucleotide positions are indicated
according to the shortest, 898-nucleotide minigenome.
20   Nucleotide lengths of specific regions are given in
parentheses. The sequence of positions 90 to 124 (3´ to 5´,
negative-sense) is shown at the bottom; PIV3-specific sequence
is underlined, the complement of a vaccinia virus
transcriptional terminator is overlined, the complement of the
25   XbaI site is italicized, and the complement of the
translational start codon for the CAT ORF is in bold. The
site where 0 to 6 G residues were inserted, resulting in
PIV3-CAT 0 to +6, is indicated. Abbreviations: GS,
transcriptional gene-start motif; GE, transcriptional gene-end
30   termination/polyadenylation motif; NT, nontranslated gene
sequence.

Figure 4 depicts expression of CAT enzyme by
minigenomes PIV3-CAT 0 to +6. Cells were transfected with
plasmids encoding the indicated PIV3-CAT minigenome and the N,
35   P and L proteins, and were infected simultaneously with
vaccinia virus recombinant vTF7-3 expressing T7 RNA
polymerase. Lysates were prepared 48 h post-infection, and
samples representing 3000 cells were analyzed for CAT

13

activity. Activities are expressed relative to the +2
minigenome, which is a multiple of six, as 100. Samples in
which the L plasmid was omitted did not have CAT activity
detectable under these conditions.

5   Figures 5A and 5B depict synthesis of
mini-antigenomic RNA by PIV3-CAT minigenomes 0 to +6. Cells
were transfected and infected as described for Fig. 4, except
for controls in which N plasmid (lane 1) or L plasmid (lane 2)
was omitted. The PIV3-CAT minigenome plasmid which was
10   transfected in each case is indicated. Cell lysates were
prepared at 48 h post-transfection: one aliquot was treated
with micrococcal nuclease (treated) and the second was
mock-treated (untreated), followed by processing for RNA
purification. The RNAs were analyzed by Northern blot
15   hybridization with a negative-sense CAT riboprobe. As a
marker and control for nuclease treatment, the RSV-CAT C2
minigenome (Grosfeld et al., J. Virol. 69:5677-5686 (1995),
incorporated herein by reference in its entirety) was
complemented by the RSV N, P and L proteins and was processed
20   in parallel (Lane 10). Autoradiograms of the hybridized blots
are shown in Fig. 5A. Fig. 5B shows phosphorimager analysis
in which the amount of hybridization relative to the +2
minigenome was calculated separately for the treated and
untreated samples.

25   Figures 6A and 6B depict synthesis of polyadenylated
mRNA by PIV3-CAT minigenomes 0 to +6. Using aliquots of cells
from the same experiment as shown in Figs. 5A and 5B, total
intracellular RNA was harvested at 48 h post-transfection and
fractionated by oligo(dT) chromatography into bound and
30   unbound fractions, which were analyzed by Northern blot
hybridization with a negative-sense CAT riboprobe. Fig. 6A
shows autoradiograms of the hybridized blots. Fig. 6B shows
the phosphorimager analysis in which the amount of
hybridization was normalized relative to the bound RNA
35   fraction of the +2 minigenome, this was calculated separately
for the bound and unbound fractions.

           Figures 7A and 7B depict accumulation of
intracellular minigenome in response to plasmid encoding

14

PIV3-CAT minigenomes 0 to +6. This is a continuation of the
experiment shown in Figs. 5A and 5B. Thus, cells which had
been transfected with plasmids encoding a minigenome and the
N, P and L proteins and infected with vTF7-3 were harvested 48

5   h post-transfection and cell lysates were prepared. One
aliquot of lysate was treated with micrococcal nuclease
(treated) and the other was mock-treated (untreated), followed
by processing for RNA purification. The RNAs were analyzed by
Northern blot hybridization with a positive-sense CAT

10   riboprobe, whereas in Fig. 5A the riboprobe was
negative-sense. Fig. 7A shows autoradiograms of the
hybridized blots. Fig. 7B shows phosphorimager analysis in
which the amount of hybridization relative to the +2
minigenome was calculated separately for the treated and

15   untreated samples.

          Figures 8-10 illustrate construction of p3/7(131)2G.
p3/7(131)2G encodes a complete positive-sense HPIV3 antigenome
and contains two G residues between the T7 promoter and
nucleotide 1 of the antigenome. The construction involved

20   modification separately of the left and right halves of cDNA
218(131) encoding the negative-sense genome. Using the same
strategy, a second cDNA, p3/7(131) was constructed which is
identical to p3/7(131)2G except that the two G residues were
omitted.

25   Figure 8 depicts construction of p(Left+2G),
performed by replacing the ribozyme and T7 transcription
terminator of p218(A*A'CC'D*E) with a T7 promoter including
two G residues. p218 (A*A'CC'D*E) was used as the template in
a PCR that amplified the left-hand 1224 nucleotides of the

30   HPIV3 genome (black rectangle labeled "PIV3seq.") using a left
hand PCR primer that introduced the T7 promoter. This PCR
fragment was cloned into the MluI-PmlI window of
p218(A*A'CC'D*E), resulting in p(Left+2G).

          Figure 9 depicts construction of p(Right+),

35   performed by replacing the T7 promoter of p(E*FF'GHIHKL*) with
a ribozyme and T7 terminator placed adjacent to PIV3
nucleotide 15642 in the positive-sense (antigenome) strand.
p(E*FF'GHIHKL*) was used as a template in a PCR that amplified

the right-hand 649 nucleotides of the HPIV3 genome (black
rectangle labeled "PIV3 seq.") using a mutagenic
oligonucleotide that added part of the delta ribozyme
(including a naturally-occurring RsrII site). This PCR

5   product was cloned into the RsrII-BssHII window of p3/7 to
yield pPIV3-3/7, thus reconstructing a complete ribozyme
flanked by the T7 terminator. The SwaI-NgoMI fragment of
pPIV3-3/7 was cloned into the SwaI-NgoMI windows of
p(E*FF'GHIHKL*), resulting in p(Right+). The locations of the

10   seven sequence markers are indicated with asterisks.

Figure 10 depicts construction of p3/7(131)2G. The
NgoMI-XhoI fragment of p(Right+) was cloned into the NgoMI-
XhoI window of p(Left+2G), resulting in p3/7(131)2G. The
positions of HPIV3 genes are indicated (not to scale). The

15   locations of sequence markers are indicated with asterisks.

Figure 11 shows sequence confirmation of a negative
sense recombinant PIV3. A 1379 bp fragment (nucleotides 7334-
8713) spanning the mutations at 7903, 7913, and 7915 was
generated by RT-PCR of RNA from infected cells and then

20   analyzed by cycle-sequencing. The mutations differentiating
recombinant PIV from JS wt are indicated by arrows. The
complete sequence from nt 7897 to 7922 is shown in the margin
next to each gel, with the three nucleotide differences
indicated in bold.

25   Figure 12 provides a map of plasmid
pTM(L)942/992/1558, which contains the PIV3 L cDNA with amino
acid substitutions at positions 942, 992, and 1558 in the L
protein sequence. The relative position of each of coding
change is indicated, together with the aa difference and the

30   naturally-occurring restriction site which was deliberately
ablated as a marker. Restriction sites used for cloning (SphI,
PinAI, BamHI and NheI) are indicated. The arrow shows the
direction of the L protein coding sequence and is numbered
according to amino acid position.

35   Figure 13 is a schematic representation of
recombinant PIV3 viruses bearing representative mutations
within the invention.

Figure 14 illustrates construction of cDNA encoding
the chimeric PIV3-PIV1 antigenome in which the PIV3 HN and F
ORFs were replaced by those of PIV1. First (starting from the
bottom left) the PIV3 F and HN genes were subcloned (as BspE-I
5   I-Xho I and EcoR I-Spe I fragments) from the full-length PIV3
cDNA clone p3/7(131)2G (Panel A) to generate pLit.PIV3.F3a and
pLit.PIV3.HN4 (Panel B), respectively. PCR mutagenesis (Panel
C) was performed to delete the complete coding regions of the
PIV3 F and HN genes and to introduce new restriction sites
10   (boxed). PIV1 F and HN cDNAs were prepared from infected-cell
RNA by RT-PCR and cloned into pLITMUS28 (Panel D). These
clones, pLit.1Fwt and pLit.1HNwt, were used as templates for
modification of the PIV1 F and HN genes at their start and
stop codons to introduce new restriction sites compatible with
15   those introduced in the PIV3 deletions (Panel E). Chimeric F
and HN genes were constructed by importing the PIV1 coding
regions into the PIV3 deletions to generate pLit.PIV3-1.Fhc
and pLit.PIV3-1.HNhc (Panel F). The chimeric F and HN were
assembled together to generate pSE.PIV3-1.hc (Panel G). The F
20   and HN chimeric segment was introduced into full-length PIV3
clone p3/7(131)2G by replacing its BspE I-Sph I fragment to
generate pFLC.2G+.hc (Panel H). The small boxes between genes
represent the gene end, intergenic, and gene start regions,
and the lines represent the non-coding regions. Shaded
25   portions are from PIV1, open boxes are from PIV3. The black
arrows depict the T7 promoter, while black boxes depict the
hepatitis delta virus (HDV) ribozyme.

Figure 15A is a diagram of the chimeric HN and F
genes of the chimeric rPIV3-1 (middle) in parallel with that
30   of rPIV3/JS (top; alternatively referred to herein as rJS and
JS wt rPIV3) and PIV1/Wash64 (bottom). The four junction
regions containing the sequence transitions from PIV3 to PIV1
are boxed and numbered I to IV. Each small box between genes
represents the gene end, intergenic, and gene start region,
35   and the lines represent the non-coding regions. RT-PCR
primers, specific to PIV3 M and L genes (primer pair A at top)
or specific to PIV1 M and HN genes (primer pair B at bottom)
used in Figure 14B, are depicted as arrows.

Figure 15B shows an evaluation of RT-PCR products generated using virion RNA (vRNA) as template and the PIV3- or PIV1-specific primer pairs described in Figure 14A. The reverse transcription step was omitted in a duplicate of each template, as indicated, to confirm that the PCR product was derived from RNA. For rPIV3-1, the PIV3-specific primer pair A gave rise to the expected 4.6 kb product, whereas primer pair B, specific to PIV-1 sequences not present in rPIV3-1, did not yield product. Positive controls using rPIV3/JS (labeled as rPIV3) or PIV1/Wash64 (labeled as PIV1) vRNA are shown in parallel.

Figure 16 represents sequences of PIV3-PIV1 junctions in the RT-PCR products of rPIV3-1 shown in Figure 14B were determined. The sequence for each of the four junction regions (Regions I-IV) is presented and aligned with the corresponding regions of rPIV3/JS (top line) and PIV1/Wash64 (bottom line), which were sequenced in parallel from RT-PCR products. Vertical bars indicate sequence identity, and the boxed regions indicate introduced mutations and restriction sites. The Gln to Glu codon change in the chimeric F gene is indicated by shaded box. Start and stop codons are underlined.

Figure 16 shows sequencing gels for region III (left) and IV (right) of RT-PCR products of rPIV3-1 compared with rPIV3 (left) or PIV1 (right). Start and stop codons are marked by a box, restriction sites are marked by arrows.

Figure 18 depicts multicycle growth of parental and chimeric PIVs in tissue culture. LLC-MK2 cell monolayers were inoculated with virus at an MOI of 0.01, and virus-infected cells were incubated at 32°C in the presence of trypsin. Tissue culture supernatants were harvested at 24 hour intervals, frozen, and analyzed in the same $TCID_{50}$ assay using hemadsorption to identify virus-infected cultures. Each point represents the mean titer of three separate cultures, with S.E. Indicated. The dotted horizontal line indicates the lower limit of viral detection.

Figure 19 illustrates introduction of the three L gene mutations of cp45 into pFLC.2G+.hc, the antigenomic cDNA

clone of the chimeric virus rPIV3-1. pTM(L)942/992/1558 (A)
is a plasmid clone of the L gene that carries the three
mutations found in cp45. The mutation at amino acid position
942 in the PIV3 L protein is a tyr (wt) to his (cp45)

5   substitution and a nearby Eae I site was ablated to mark this
site. Similarly, the 992 mutation is a leu to phe change with
a Bsr I site ablated and the 1558 mutation is a thr to ile
change with an Ava II site ablated. The 2.9 kb SphI-NheI
fragment present in pTM(L)942/992/1558 was introduced into

10   pFLC.2G+hc (B), the plasmid carrying the full length cDNA
clone of rPIV3-1, to give pFLC+hc.cp45L (C). For the
constructs in (B) and (C), the black boxes indicate the
location of the hepatitis D virus ribozyme and the T7
teminator, the shaded regions are the PIV1 HN and F ORFs, and

15   the open boxes represent sequences derived from PIV3.
pFLC.2G+.hc.cp45L was used in the transfection to yield the
attenuated chimeric recombinant virus designated rPIV3-
1.cp45L.

20   Description of the Specific Embodiments
        The present invention provides compositions and
methods for producing and modifying infectious PIV from
isolated polynucleotide molecules, preferably cDNA.
Infectious PIV particles are produced by a recombinant

25   coexpression system that permits introduction of defined
changes into infectious PIV. These modifications are useful
in a wide variety of applications, including the development
of live attenuated vaccine strains bearing predetermined,
defined attenuating mutations.

30   Infectious PIV of the invention are produced by
intracellular or cell-free coexpression of one or more
isolated polynucleotide molecules that encode a PIV genome or
antigenome RNA, together with one or more polynucleotides
encoding viral proteins necessary to generate a transcribing,

35   replicating nucleocapsid. Among the viral proteins useful for
coexpression to yield infectious PIV are the major
nucleocapsid protein (N) protein, nucleocapsid phosphoprotein
(P), large (L) polymerase protein, fusion protein (F),

hemagglutinin-neuraminidase glycoprotein (HN), and matrix (M)
protein. Also useful in this context are products of the C, D
and V ORFs of PIV.

5   cDNAs encoding a PIV genome or antigenome are
constructed for intracellular or *in vitro* coexpression with
the necessary viral proteins to form infectious PIV. By "PIV
antigenome" is meant an isolated positive-sense polynucleotide
molecule which serves as a template for synthesis of progeny
PIV genome. Preferably a cDNA is constructed which is a
10   positive-sense version of the PIV genome corresponding to the
replicative intermediate RNA, or antigenome, so as to minimize
the possibility of hybridizing with positive-sense transcripts
of complementing sequences encoding proteins necessary to
generate a transcribing, replicating nucleocapsid.

15   In some embodiments of the invention the genome or
antigenome of a recombinant PIV (rPIV) need only contain those
genes or portions thereof necessary to render the viral or
subviral particles encoded thereby infectious. Further, the
genes or portions thereof may be provided by more than one
20   polynucleotide molecule, i.e., a gene may be provided by
complementation or the like from a separate nucleotide
molecule. In other embodiments, the PIV genome or antigenome
encodes all functions necessary for viral growth, replication,
and infection without the participation of a helper virus or
25   viral function provided by a plasmid or helper cell line.

By "recombinant PIV" is meant a PIV or PIV-like
viral or subviral particle derived directly or indirectly from
a recombinant expression system or propagated from virus or
subviral particles produced therefrom. The recombinant
30   expression system will employ a recombinant expression vector
which comprises an operably linked transcriptional unit
comprising an assembly of at least a genetic element or
elements having a regulatory role in PIV gene expression, for
example, a promoter, a structural or coding sequence which is
35   transcribed into PIV RNA, and appropriate transcription
initiation and termination sequences.

To produce infectious PIV from a cDNA-expressed PIV
genome or antigenome, the genome or antigenome is coexpressed

20

with those PIV N, P and L proteins necessary to (i) produce a
nucleocapsid capable of RNA replication, and (ii) render
progeny nucleocapsids competent for both RNA replication and
transcription. Transcription by the genome nucleocapsid

5   provides the other PIV proteins and initiates a productive
infection. Alternatively, additional PIV proteins needed for
a productive infection can be supplied by coexpression.

        Synthesis of PIV antigenome or genome together with
the above-mentioned viral proteins can also be achieved *in*

10   *vitro* (cell-free), e.g., using a combined transcription-
translation reaction, followed by transfection into cells.
Alternatively, antigenome or genome RNA can be synthesized *in*
vitro and transfected into cells expressing PIV proteins.

        In certain embodiments of the invention,

15   complementing sequences encoding proteins necessary to
generate a transcribing, replicating PIV nucleocapsid are
provided by one or more helper viruses. Such helper viruses
can be wild type or mutant. Preferably, the helper virus can
be distinguished phenotypically from the virus encoded by the

20   PIV cDNA. For example, it is desirable to provide monoclonal
antibodies which react immunologically with the helper virus
but not the virus encoded by the PIV cDNA. Such antibodies
can be neutralizing antibodies. In some embodiments, the
antibodies can be used in affinity chromatography to separate

25   the helper virus from the recombinant virus. To aid the
procurement of such antibodies, mutations can be introduced
into the PIV cDNA to provide antigenic diversity from the
helper virus, such as in the HN or F glycoprotein genes.

        In alternate embodiments of the invention, the N, P,

30   L and other desired PIV proteins are encoded by one or more
non-viral expression vectors, which can be the same or
separate from that which encodes the genome or antigenome.
Additional proteins may be included as desired, each encoded
by its own vector or by a vector encoding one or more of the

35   N, P, L and other desired PIV proteins, or the complete genome
or antigenome. Expression of the genome or antigenome and
proteins from transfected plasmids can be achieved, for
example, by each cDNA being under the control of a promoter

for T7 RNA polymerase, which in turn is supplied by infection, transfection or transduction with an expression system for the T7 RNA polymerase, e.g., a vaccinia virus MVA strain recombinant which expresses the T7 RNA polymerase (Wyatt et al., *Virology*, 210: 202-205 (1995), incorporated herein by reference in its entirety). The viral proteins, and/or T7 RNA polymerase, can also be provided by transformed mammalian cells or by transfection of preformed mRNA or protein.

A PIV antigenome may be constructed for use in the present invention by, e.g., assembling cloned cDNA segments, representing in aggregate the complete antigenome, by polymerase chain reaction or the like (PCR; described in, e.g., U.S. Patent Nos. 4,683,195 and 4,683,202, and *PCR Protocols: A Guide to Methods and Applications*, Innis et al., eds., Academic Press, San Diego (1990), each incorporated herein by reference in its entirety) of reverse-transcribed copies of PIV mRNA or genome RNA. For example, a first construct is generated which comprises cDNAs containing the left hand end of the antigenome, spanning from an appropriate promoter (e.g., T7 RNA polymerase promoter) and assembled in an appropriate expression vector, such as a plasmid, cosmid, phage, or DNA virus vector. The vector may be modified by mutagenesis and/or insertion of synthetic polylinker containing unique restriction sites designed to facilitate assembly. For ease of preparation the N, P, L and other desired PIV proteins can be assembled in one or more separate vectors. The right hand end of the antigenome plasmid may contain additional sequences as desired, such as a flanking ribozyme and tandem T7 transcriptional terminators. The ribozyme can be hammerhead type (e.g., Grosfeld et al., (1995), *supra*), which would yield a 3' end containing a single nonviral nucleotide, or can be any of the other suitable ribozymes such as that of hepatitis delta virus (Perrotta et al., *Nature* 350:434-436 (1991), incorporated herein by reference in its entirety) which would yield a 3' end free of non-PIV nucleotides. The left- and righthand ends are then joined via a common restriction site.

A variety of nucleotide insertions, deletions and rearrangements can be made in the PIV genome or antigenome during or after construction of the cDNA. For example, specific desired nucleotide sequences can be synthesized and inserted at appropriate regions in the cDNA using convenient restriction enzyme sites. Alternatively, such techniques as site-specific mutagenesis, alanine scanning, PCR mutagenesis, or other such techniques well known in the art can be used to introduce mutations into the cDNA.

Alternative means to construct cDNA encoding the genome or antigenome include reverse transcription-PCR using improved PCR conditions (e.g., as described in Cheng et al., Proc. Natl. Acad. Sci. USA 91:5695-5699 (1994)), incorporated herein by reference) to reduce the number of subunit cDNA components to as few as one or two pieces. In other embodiments different promoters can be used (e.g., T3, SP6) or different ribozymes (e.g., that of hepatitis delta virus. Different DNA vectors (e.g., cosmids) can be used for propagation to better accommodate the larger size genome or antigenome.

Isolated polynucleotides (e.g., cDNA) encoding the genome or antigenome may be inserted into appropriate host cells by transfection, electroporation, mechanical insertion, transduction or the like, into cells which are capable of supporting a productive PIV infection, e.g., HEp-2, FRhL-DBS2, LLC-MK2, MRC-5, and Vero cells. Transfection of isolated polynucleotide sequences may be introduced into cultured cells by, for example, calcium phosphate-mediated transfection (Wigler et al., Cell 14: 725 (1978); Corsaro and Pearson, Somatic Cell Genetics 7: 603 (1981); Graham and Van der Eb, Virology 52: 456 (1973)), electroporation (Neumann et al., EMBO J. 1: 841-845 (1982)), DEAE-dextran mediated transfection (Ausubel et al., (ed.) Current Protocols in Molecular Biology, John Wiley and Sons, Inc., NY (1987), cationic lipid-mediated transfection (Hawley-Nelson et al., Focus 15: 73-79 (1993)) or a commercially available transfection regent, e.g., LipofectACE® (Life Technologies, Gaithersburg, MD) or the like

23

(each of the foregoing references are incorporated herein by
reference in its entirety).

As noted above, in some embodiments of the invention
the N, P, L and other desired PIV proteins are encoded by one
5   or more helper viruses which is phenotypically distinguishable
from that which encodes the genome or antigenome. The N, P, L
and other desired PIV proteins can also be encoded by one or
more expression vectors which can be the same or separate from
that which encodes the genome or antigenome, and various
10   combinations thereof. Additional proteins may be included as
desired, encoded by its own vector or by a vector encoding one
or more of the N, P, L and other desired PIV proteins, or the
complete genome or antigenome.

The compositions and methods of the invention permit
15   analysis and manipulation of PIV molecular biology and
pathogenic mechanisms using, e.g., defined mutations to alter
the function or expression of selected PIV proteins. Using
these methods and compositions, one can readily distinguish
mutations responsible for desired phenotypic changes from
20   silent incidental mutations, and select phenotype-specific
mutations for incorporation into a recombinant PIV genome or
antigenome for vaccine production.

Modifications of PIV provided within the invention
are directed toward the production of improved vaccine
25   viruses, e.g., to enhance viral attenuation and vaccine
immunogenicity, to ablate epitopes associated with undesirable
immunopathology, to accommodate antigenic drift, etc. To
achieve these and other objectives, the compositions and
methods of the invention allow for a wide variety of
30   modifications to be introduced into a PIV genome or antigenome
for incorporation into infectious, recombinant PIV. For
example, foreign genes or gene segments encoding protective
antigens or epitopes may be added within a PIV clone to
generate PIV virus strains capable of inducing immunity to
35   both PIV and another virus or agent from which the protective
antigen was derived. Alternatively, foreign genes may be
inserted, in whole or in part, encoding modulators of the

immune system, such as cytokines, to enhance immunogenicity of
a vaccine virus.

Other mutations which may be included within PIV
clones of the invention include, for example, substitution of
heterologous genes or gene segments (e.g., a gene segment
encoding a cytoplasmic tail of a glycoprotein gene) with a
counterpart gene or gene segment in a PIV clone.
Alternatively, the relative order of genes within a PIV clone
can be changed, the PIV genome promoter can be replaced with
its antigenome counterpart, or selected gene(s) rendered non-
functional (e.g., by functional ablation involving
introduction of a stop codon to prevent expression of the
gene). Other modifications in a PIV clone can be made to
facilitate manipulations, such as the insertion of unique
restriction sites in various non-coding or coding regions or
elsewhere. In addition, nontranslated gene sequences can be
removed to increase capacity for inserting foreign sequences.

Thus, by providing infectious clones of PIV the
invention permits a wide range of alterations to be
recombinantly produced within the PIV genome (or antigenome),
yielding defined mutations which specify desired phenotypic
changes. By "infectious clone" is meant cDNA or its product,
synthetic or otherwise, RNA capable of being directly
incorporated into infectious virions which can be transcribed
into genomic or antigenomic RNA capable of serving as a
template to produce the genome of infectious viral or subviral
particles. As noted above, defined mutations can be
introduced by a variety of conventional techniques (e.g.,
site-directed mutagenesis) into a cDNA copy of the genome or
antigenome. The use of genomic or antigenomic cDNA
subfragments to assemble a complete genome or antigenome cDNA
as described herein has the advantage that each region can be
manipulated separately, where small cDNA subjects provide for
better ease of manipulation than large cDNA subjects, and then
readily assembled into a complete cDNA. Thus, the complete
antigenome or genome cDNA, or a selected subfragment thereof,
can be used as a template for oligonucleotide-directed
mutagenesis. This can be through the intermediate of a

single-stranded phagemid form, such as using the MUTA-gen® kit
of Bio-Rad Laboratories (Richmond, CA), or a method using the
double-stranded plasmid directly as a template such as the
Chameleon® mutagenesis kit of Strategene (La Jolla, CA), or by
5   the polymerase chain reaction employing either an
oligonucleotide primer or a template which contains the
mutation(s) of interest. A mutated subfragment can then be
assembled into the complete antigenome or genome cDNA. A
variety of other mutagenesis techniques are known and can be
10   routinely adapted for use in producing the mutations of
interest in a PIV antigenome or genome cDNA of the invention.
    Thus, in one illustrative embodiment mutations are
introduced by using the MUTA-gene® phagemid _in vitro_
mutagenesis kit available from Bio-Rad Laboratories. In
15   brief, cDNA encoding an PIV genome or antigenome is cloned
into the plasmid pTZ18U, and used to transform CJ236 cells
(Life Technologies). Phagemid preparations are prepared as
recommended by the manufacturer. Oligonucleotides are
designed for mutagenesis by introduction of an altered
20   nucleotide at the desired position of the genome or
antigenome. The plasmid containing the genetically altered
genome or antigenome is then amplified.
    Mutations can vary from single nucleotide changes to
the introduction, deletion or replacement of large cDNA
25   segments containing one or more genes or genome segments.
Genome segments can correspond to structural and/or functional
domains, e.g., cytoplasmic, transmembrane or ectodomains of
proteins, active sites such as sites that mediate binding or
other biochemical interactions with different proteins,
30   epitopic sites, e.g., sites that stimulate antibody binding
and/or humoral or cell mediated immune responses, etc. Useful
genome segments in this regard range from about 15-35
nucleotides in the case of genome segments encoding small
functional domains of proteins, e.g., epitopic sites, to about
35   50, 75, 100, 200-500, and 500-1,500 or more nucleotides.
    The ability to introduce defined mutations into
infectious PIV has many applications, including the
manipulation of PIV pathogenic and immunogenic mechanisms.

For example, the functions of PIV proteins, including the N,
P, M, F, HN, and L proteins and C, D and V ORF products, can
be manipulated by introducing mutations which ablate or reduce
the level of protein expression, or which yield mutant
5   protein. In one such exemplary modification, a sequence at
the cleavage site of the F protein can be modified and the
effects of this modification on growth in tissue culture and
infection and pathogenesis of the resultant PIV can be
routinely determined in experimental animals.
10   Various genome RNA structural features, such as
promoters, intergenic regions, and transcription signals, can
also be routinely manipulated within the methods and
compositions of the invention. The effects of trans-acting
proteins and cis-acting RNA sequences can be readily
15   determined, for example, using a complete antigenome cDNA in
parallel assays employing PIV minigenomes (Dimock, et al., _J._
Virol. _67_: 2772-8 (1993), incorporated herein by reference in
its entirety), whose rescue-dependent status is useful in
characterizing those mutants that may be too inhibitory to be
20   recovered in replication-independent infectious virus.
            The present invention further provides tools and
methods to readily distinguish between silent incidental
mutations and mutations responsible for phenotype differences,
for example by introducing suspect mutations, separately and
25   in various combinations, into the genome or antigenome of
infectious wild-type (i.e., for one or more phenotypic
character such as temperature sensitivity, replication in a
selected host, etc.) PIV. This process permits identification
of mutations responsible for desired vaccine phenotypes such
30   as attenuation, temperature sensitivity, cold-adaptation,
small plaque size, host range restriction, etc. Mutations
identified by these methods can then be introduced in various
combinations to modify a vaccine virus to an appropriate level
of attenuation, etc., as desired. Moreover, the present
35   invention provides the ability to combine mutations from
different strains of virus into a single vaccine strain.
            As noted above, mutations incorporated within
recombinantly altered PIV clones may be selected based on

their ability to alter expression and/or function of a
selected PIV protein, yielding a desired phenotypic change, or
for a variety of other purposes. Desired phenotypic changes
include, e.g., changes in viral growth in culture, temperature
sensitivity, plaque size, attenuation, and immunogenicity.
For example, a polynucleotide sequence encoding the genome or
antigenome can be modified by a nucleotide insertion,
rearrangement, deletion or substitution to specify
attenuation, temperature-sensitivity, cold-adaptation, small
plaque size, host range restriction, alteration in gene
expression, or a change in an immunogenic epitope.

In one aspect of the invention, mutations occurring
in biologically derived, attenuated PIV are identified and
introduced individually or in combination into a full-length
PIV clone, and the phenotypes of rescued recombinant viruses
containing the introduced mutations are determined. In
exemplary embodiments, amino acid changes displayed by
biologically derived mutant viruses over a wild-type PIV, for
example changes exhibited by PIV mutants having *ts*, *ca* or *att*
phenotypes, are incorporated within recombinant PIV clones.
These changes from biologically derived mutant PIV specify
desired characteristics in the resultant clones, e.g., an
attenuation phenotype specified by a mutation adopted from the
HPIV3 mutant JS *cp*45. These changes are preferably introduced
into recombinant virus using two or three nucleotide changes
compared to a corresponding wild type or biologically derived
mutant sequence, which has the effect of stabilizing the
mutation against genetic reversion.

The present invention also provides recombinant PIV
having multiple, phenotype-specifying mutations introduced in
selected combinations into the genome or antigenome of an
infectious clone. This process, coupled with evaluation of
phenotype, provides mutant recombinant PIV having such desired
characteristics as attenuation, temperature sensitivity,
cold-adaptation, small plaque size, host range restriction,
etc. Thus, exemplary PIV clones are disclosed herein which
incorporate one or more, and preferably at least two
attenuating mutations, e.g., *ts*, *ca* or *att* mutations adopted

from a biologically derived PIV mutant, such as JS *cp*45.
Target genes for adopting biologically derived mutations in a
recombinant PIV in this context include the nucleocapsid
protein N, phosphoprotein P, large polymerase subunit L,

5   matrix protein M, hemagglutinin-neuraminidase protein HN,
fusion protein F and the C, D and V ORF products. Also
targeted are extragenic sequences, eg., sequences in the 3'
leader or trailer regions of a PIV genome, and in cis-acting
elements such as gene start and gene end sequences, eg., the N

10   gene start signal. Exemplary mutations incorporated in
recombinant PIV herein include one or more nucleotide
substitutions specifying amino acid change(s) in the
polymerase L gene, e.g., at Tyr_{942}, Leu_{992}, and/or Thr_{1558}. For
example, PIV recombinants are disclosed wherein Tyr_{942} is

15   replaced by His, Leu_{992} is replaced by Phe, and/or Thr_{1558} is
replaced by Ile. These mutations have been successfully
incorporated in various exemplary PIV recombinants herein,
including r942, r992, r1558, r942/992, r992/1558, r942/1558,
or r942/992/1558 recombinants described in the Examples below.

20   Other exemplary mutations adopted from a biologically derived
PIV mutant include one or more mutations in the N protein,
including specific mutations at a position corresponding to
residues Val_{96} or Ser_{389} of JS *cp*45. In more detailed aspects,
these mutations are represented as Val_{96} to Ala or Ser_{389} to

25   Ala. Also disclosed in the Examples below are recombinant PIV
which encode an amino acid substitution in the C protein, eg.,
a mutation at a position corresponding to Ile_{96} of JS *cp*45,
preferably represented by a substitution of Ile_{96} to Thr.
Further exemplary mutations adopted from biologically derived

30   PIV mutants include one or more mutations in the F protein,
including mutations adopted from JS *cp*45 at a position
corresponding to residues Ile_{420} or Ala_{450} of JS *cp*45,
preferably represented by acid substitutions Ile_{420} to Val or
Ala_{450} to Thr. Other PIV recombinants within the invention

35   adopt one or more amino acid substitutions in the HN protein,
as exemplified hereinbelow by a recombinant PIV adopting a
mutation at a position corresponding to residue Val_{384} of JS
*cp*45, preferably represented by the substitution Val_{384} to Ala.

Yet additional examples within this aspect of the invention
include recombinant PIV which incorporate one or more
mutations in an extragenic sequence, eg., a 3' leader sequence
of recombinant PIV genome or antigenome. Exemplary mutations
5   in this context include mutations in the 3' leader occurs at
one or more positions corresponding to nucleotide 23, 24,
and/or 28 of JS cp45, for example a T to C change at
nucleotide 23, a C to T change at nucleotide 24, or a G to T
change at nucleotide 28. Yet additional extragenic mutations
10   include one or more mutations in a N gene start sequence, as
exemplified hereinbelow by a mutation in the N gene start
sequence at a position corresponding to nucleotide 62 of JS
cp45, preferably represented by a A to T change. The above
exemplary mutations adopted from biologically derived mutant
15   PIV are evaluated and combined into recombinant PIV in the
Examples below to result, individually and/or in combination,
in novel, attenuated candidate vaccine strains, as exemplified
by the recombinants designated herein as rcp45, rcp45
3'NCMFHN, rcp45 3'NL, rcp45 3'N, and rcp45 F. Other PIV
20   recombinants within the invention will incorporate a plurality
and up to a full complement of the mutations present in one or
more of these exemplary recombinants, as well as mutations
identified in other biologically derived mutant PIV strains
identified and adopted in a recombinant PIV according to the
25   teachings herein.

       Mutations identified according to the methods
disclosed herein are compiled into a "menu" and introduced in
various combinations to calibrate a vaccine virus to a
selected level of attenuation, immunogenicity and stability.
30   In preferred embodiments, the invention provides for
supplementation of one or more mutations adopted from
biologically derived PIV, e.g., ts, ca or att mutations, with
additional types of mutations involving the same or different
genes. Target genes for mutation in this context also include
35   the nucleocapsid protein N, phosphoprotein P, large polymerase
subunit L, matrix protein M, hemagglutinin-neuraminidase
protein HN, fusion protein F and the C, D and V ORF products.
In one aspect, recombinant PIVs are provided wherein at least

one attenuating mutation occurs in the PIV polymerase gene L
and involves a nucleotide substitution specifying a *ts* or *att*
phenotype adopted from a biologically derived mutant PIV
strain, for example JS *cp*45. Exemplary HPIV3 recombinants

5   disclosed herein include the r942, r992, r1558, r942/992,
r992/1558, r942/1558, or r942/992/1558 recombinants described
in the Examples below. These exemplary PIV clones incorporate
one or more nucleotide substitutions resulting in an amino
acid change in the polymerase gene, e.g., at Tyr_{942}, Leu_{992},

10   and/or Thr_{1558}. For example, PIV recombinants are disclosed
wherein Tyr_{942} is replaced by His, Leu_{992} is replaced by Phe,
and/or Thr_{1558} is replaced by Ile. Preferably, two or three
mutations are incorporated in a codon specifying an
attenuating mutation adding increased stability against

15   phenotypic reversion.

In additional aspects, a variety of other genetic
alterations can be produced in a recombinant PIV genome or
antigenome for incorporation into infectious recombinant PIV,
alone or together with one or more attenuating mutations

20   adopted from a biologically derived mutant PIV. Heterologous
genes (e.g. from different PIV strains or non-PIV sources such
as another virus, e.g., RSV or measles virus) may be inserted
or substituted, in whole or in part, the order of genes
changed, gene overlap removed, the PIV genome promoter

25   replaced with its antigenome counterpart, and even entire,
non-essential genes deleted. In one aspect, a selected PIV
gene, for example the C, D, or V ORF, is functionally deleted
to yield a recombinant PIV having novel phenotypic
characteristics, for example enhanced growth *in vitro* and/or

30   attenuation *in vivo*. An infectious PIV clone of the invention
can also be engineered to enhance its immunogenicity and
induce a level of protection greater than that provided by
natural infection, or to ablate epitopes associated with
undesirable immunopathologic reactions. Enhanced

35   immunogenicity of the vaccines produced by the present
invention addresses one of the greatest obstacles to
controlling PIV, namely the incomplete nature of immunity
induced by natural infection. In this context, additional

gene(s) or gene segment(s) may be inserted into or proximate
to the PIV genome or antigenome which may be placed under the
control of a common or independent set of transcription
signals. Genes of interest include those encoding cytokines

5   (e.g., IL-2 through IL-15, especially IL-2, IL-6 and IL-12,
etc.) and proteins rich in T helper cell epitopes. The
additional protein can be expressed either as a separate
protein or as a chimera engineered from a second copy of one
of the PIV proteins, such as HN. This provides the ability to

10   modify and improve the immune response against PIV both
quantitatively and qualitatively.

       Other mutations useful within the invention involve
replacement of the 3' end of genome with its counterpart from
antigenome, which is associated with changes in RNA

15   replication and transcription. In addition, intergenic
regions can be shortened or lengthened or changed in sequence
content. In yet additional aspects, PIV useful in a vaccine
formulation can be conveniently modified to accommodate
antigenic drift in circulating virus. Typically the

20   modification will be in the HN and/or F proteins. For
example, a selected antigenic form of an entire HN or F gene,
or the segment(s) encoding particular immunogenic regions
thereof, is incorporated into a PIV genome or antigenome cDNA
by replacement of a counterpart region in the infectious

25   clone, or by adding one or more copies of the gene such that
several antigenic forms are represented in the resultant
clone. Progeny virus produced from the modified PIV cDNA are
then used in vaccination protocols against emerging strains.

       Other mutations for use in infectious PIV of the

30   invention include mutations in cis-acting signals identified
during mutational analysis of PIV minigenomes. For example,
insertional and deletional analysis of leader and trailer and
flanking sequences identify viral promoters and transcription
signals and provide a series of mutations associated with

35   varying degrees of reduction of RNA replication or
transcription. Saturation mutagenesis (whereby each position
in turn is modified to each of the nucleotide alternatives) of
these cis-acting signals also identifies mutations which

reduce or increase RNA replication or transcription. Any of
these mutations can be inserted into the complete antigenome
or genome as described herein.

Additional modifications in PIV clones can be made
5   to facilitate manipulations, such as the insertion of unique
restriction sites in various intergenic regions or elsewhere.
Nontranslated gene sequences can also be removed to increase
capacity for inserting foreign sequences.

Certain substitutions, insertions, deletions or
10   rearrangements of genes or gene segments within recombinant
PIV of the invention (e.g., substitutions of a gene segment
encoding a selected protein or protein region, for instance a
cytoplasmic tail, transmembrane domain or ectodomain, an
epitopic site or region, a binding site or region, an active
15   site or region containing an active site, etc.) are made in
structural or functional relation to an existing,
"counterpart" gene or gene segment from the same or different
PIV or other source. Such modifications yield novel
recombinants having desired phenotypic changes compared to
20   wild-type or parental PIV or other viral strains. For
example, recombinants of this type may express a chimeric
protein having a cytoplasmic tail and/or transmembrane domain
of one PIV fused to an ectodomain of another PIV. Other
exemplary recombinants of this type express duplicate protein
25   regions, such as duplicate immunogenic regions.

As used herein, "counterpart" genes, gene segments,
proteins or protein regions, are typically from heterologous
sources (e.g., from different PIV genes, or representing the
same (i.e., homologous or allelic) gene or gene segment in
30   different PIV types or strains). Typical counterparts
selected in this context share gross structural features,
e.g., each counterpart may encode a comparable protein or
protein structural domain, such as a cytoplasmic domain,
transmembrane domain, ectodomain, binding site or region,
35   epitopic site or region, etc. Counterpart domains and their
encoding gene segments embrace an assemblage of species having
a range of size and sequence variations defined by a common
biological activity among the domain or gene segment variants.

For example, two selected protein domains encoded by
counterpart gene segments within the invention share
substantially the same qualitative activity, such as providing
a membrane spanning function, a specific binding activity, an
immunological recognition site, etc. More typically, a
specific biological activity shared between counterparts,
e.g., between selected protein segments or proteins, will be
substantially similar quantitatively, i.e., they will not vary
in respective quantitative activity levels by more than 30%,
preferably by no more than 20%, more preferably by no more
than 5-10%.

Counterpart genes and gene segments, as well as
other polynucleotides disclosed herein for producing
recombinant PIV within the invention, preferably share
substantial sequence identity with a selected polynucleotide
"reference sequence," e.g., with another selected counterpart
sequence. As used herein, a "reference sequence" is a defined
sequence used as a basis for sequence comparison, for example,
a segment of a full-length cDNA or gene, or a complete cDNA or
gene sequence. Generally, a reference sequence is at least 20
nucleotides in length, frequently at least 25 nucleotides in
length, and often at least 50 nucleotides in length. Since
two polynucleotides may each (1) comprise a sequence (i.e., a
portion of the complete polynucleotide sequence) that is
similar between the two polynucleotides, and (2) may further
comprise a sequence that is divergent between the two
polynucleotides, sequence comparisons between two (or more)
polynucleotides are typically performed by comparing sequences
of the two polynucleotides over a "comparison window" to
identify and compare local regions of sequence similarity. A
"comparison window", as used herein, refers to a conceptual
segment of at least 20 contiguous nucleotide positions wherein
a polynucleotide sequence may be compared to a reference
sequence of at least 20 contiguous nucleotides and wherein the
portion of the polynucleotide sequence in the comparison
window may comprise additions or deletions (i.e., gaps) of 20
percent or less as compared to the reference sequence (which
does not comprise additions or deletions) for optimal

alignment of the two sequences. Optimal alignment of
sequences for aligning a comparison window may be conducted by
the local homology algorithm of Smith & Waterman, Adv. Appl.
Math. 2:482 (1981), by the homology alignment algorithm of
5   Needleman & Wunsch, J. Mol. Biol. 48:443 (1970), by the search
for similarity method of Pearson & Lipman, Proc. Natl. Acad.
Sci. USA 85:2444 (1988) (each of which is incorporated by
reference), by computerized implementations of these
algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin
10   Genetics Software Package Release 7.0, Genetics Computer
Group, 575 Science Dr., Madison, WI, incorporated herein by
reference), or by inspection, and the best alignment (i.e.,
resulting in the highest percentage of sequence similarity
over the comparison window) generated by the various methods
15   is selected. The term "sequence identity" means that two
polynucleotide sequences are identical (i.e., on a nucleotide-
by-nucleotide basis) over the window of comparison. The term
"percentage of sequence identity" is calculated by comparing
two optimally aligned sequences over the window of comparison,
20   determining the number of positions at which the identical
nucleic acid base (e.g., A, T, C, G, U, or I) occurs in both
sequences to yield the number of matched positions, dividing
the number of matched positions by the total number of
positions in the window of comparison (i.e., the window size),
25   and multiplying the result by 100 to yield the percentage of
sequence identity. The terms "substantial identity" as used
herein denotes a characteristic of a polynucleotide sequence,
wherein the polynucleotide comprises a sequence that has at
least 85 percent sequence identity, preferably at least 90 to
30   95 percent sequence identity, more usually at least 99 percent
sequence identity as compared to a reference sequence over a
comparison window of at least 20 nucleotide positions,
frequently over a window of at least 25-50 nucleotides,
wherein the percentage of sequence identity is calculated by
35   comparing the reference sequence to the polynucleotide
sequence which may include deletions or additions which total
20 percent or less of the reference sequence over the window

of comparison. The reference sequence may be a subset of a
larger sequence.

In addition to these polynucleotide sequence
relationships, proteins and protein regions encoded by
5   recombinant PIV of the invention are also typically selected
to have conservative relationships, i.e. to have substantial
sequence identity or sequence similarity, with selected
reference polypeptides. As applied to polypeptides, the term
"sequence identity" means peptides share identical amino acids
10   at corresponding positions. The term "sequence similarity"
means peptides have identical or similar amino acids (i.e.,
conservative substitutions) at corresponding positions. The
term "substantial sequence identity" means that two peptide
sequences, when optimally aligned, such as by the programs GAP
15   or BESTFIT using default gap weights, share at least 80
percent sequence identity, preferably at least 90 percent
sequence identity, more preferably at least 95 percent
sequence identity or more (e.g., 99 percent sequence
identity). The term "substantial similarity" means that two
20   peptide sequences share corresponding percentages of sequence
similarity. Preferably, residue positions which are not
identical differ by conservative amino acid substitutions.
Conservative amino acid substitutions refer to the
interchangeability of residues having similar side chains.
25   For example, a group of amino acids having aliphatic side
chains is glycine, alanine, valine, leucine, and isoleucine; a
group of amino acids having aliphatic-hydroxyl side chains is
serine and threonine; a group of amino acids having amide-
containing side chains is asparagine and glutamine; a group of
30   amino acids having aromatic side chains is phenylalanine,
tyrosine, and tryptophan; a group of amino acids having basic
side chains is lysine, arginine, and histidine; and a group of
amino acids having sulfur-containing side chains is cysteine
and methionine. Preferred conservative amino acids
35   substitution groups are: valine-leucine-isoleucine,
phenylalanine-tyrosine, lysine-arginine, alanine-valine, and
asparagine-glutamine. Abbreviations for the twenty naturally
occurring amino acids used herein follow conventional usage

(Immunology - A Synthesis (2nd ed., E.S. Golub & D.R. Gren, eds., Sinauer Associates, Sunderland, MA, 1991), incorporated herein by reference). Stereoisomers (e.g., D-amino acids) of the twenty conventional amino acids, unnatural amino acids

5   such as α,α-disubstituted amino acids, N-alkyl amino acids, lactic acid, and other unconventional amino acids may also be suitable components for polypeptides of the present invention. Examples of unconventional amino acids include: 4-hydroxyproline, γ-carboxyglutamate, ε-N,N,N-trimethyllysine,

10   ε-N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, ω-N-methylarginine, and other similar amino acids and imino acids (e.g., 4-hydroxyproline). Moreover, amino acids may be modified by glycosylation, phosphorylation and the like.

15   The infectious PIV produced from cDNA-expressed genome or antigenome can be any of the PIV or PIV-like strains, e.g., human, bovine, murine, etc. To engender a protective immune response, the PIV strain may be one which is endogenous to the subject being immunized, such as human PIV

20   being used to immunize humans. The genome or antigenome can be modified, however, to express heterologous PIV genes or gene segments, or genes or gene segments from other heterologous sources, e.g., RSV or measles virus. Thus, infectious PIV intended for administration to humans may be

25   human PIV that has been modified to contain genes or gene segments from a bovine or murine PIV type such as for the purpose of attenuation. BPIV3 possesses host range mutations that restrict its replication in rhesus monkeys and humans (Karron et al., supra, 1995a; van Wyke Coelingh et al., 1988),

30   each incorporated herein by reference in its entirety). Gene(s), mutations and cis-acting regulatory sequences of BPIV3 that specify desired phenotypes, e.g., host range restriction, will be identified by their substitution for corresponding sequences in rPIV of the invention, and

35   incorporated within further modified rPIV to develop yet additional useful vaccine agents. Similarly, mutations of JS cp45 which are known to impart non-ts host-range attenuating mutations for the rhesus monkey (Hall et al., supra, (1992))

will likewise be identified and incorporated into modified
rPIV vaccine agents of the invention. Alternatively, a bovine
PIV may be modified to contain genes that encode, e.g.,
proteins or immunogenic epitopes that elicit protection
5   against human PIV infection. For example, human PIV
glycoprotein genes can be substituted for counterpart bovine
glycoprotein genes, such that the bovine PIV elicits a
protective immune response in humans against human PIV
strains.

10   In exemplary embodiments, individual genes, gene
segments, or single or multiple nucleotides of one PIV are
substituted by counterpart sequence(s) from a heterologous PIV
or other source. For example, heterologous gene segments,
such as one encoding a cytoplasmic tail, transmembrane domain
15   or ectodomain, an epitopic site or region, a binding site or
region, an active site or region containing an active site,
etc., of a selected protein from one PIV is substituted for a
counterpart gene segment in another PIV to yield novel
recombinants, for example recombinants expressing a chimeric
20   protein having a cytoplasmic tail and/or transmembrane domain
of one PIV fused to an ectodomain of another PIV. Preferred
genome segments in this regard range from about 15-35
nucleotides in the case of gene segments encoding small
functional domains of proteins, e.g., epitopic sites, to about
25   50, 75, 100, 200-500, or 500-1,500 or more nucleotides for
gene segments encoding larger domains or protein regions.

          In one aspect of the invention, selected domains of
HN and/or F proteins of one PIV strain are substituted into a
heterologous PIV clone to produce a recombinant virus capable
30   of stimulating a cross-protective immune response against both
PIV strains in an immunized host. In other aspects, modified
PIV clones are provided which comprise a chimera of a human
PIV genomic or antigenomic sequence and at least one non-human
PIV sequence, for example a polynucleotide containing
35   sequences from both human and bovine PIV. The replacement of
a human PIV coding sequence or non-coding sequence (e.g., a
promoter, gene-end, gene-start, intergenic or other cis-acting
element) with a counterpart bovine or murine PIV sequence

38

yields recombinants having a variety of possible attenuating effects. For example, a host range effect will often arise from a heterologous PIV gene not functioning efficiently in a human cell, from incompatibility of the heterologous sequence
5   or protein with a biologically interactive human PIV sequence or protein (e.g., a sequence or protein that ordinarily cooperates with the substituted sequence or protein for viral transcription, translation, assembly, etc.), among other useful attenuating effects. In yet another aspect of the
10   invention, insertion of foreign genes or gene segments, and in some cases of noncoding nucleotide sequences, into the PIV genome results in a desired increase in genome length causing yet additional, desired phenotypic effects. Increased genome length is expected to result in attenuation of the resultant
15   PIV clone, dependent in part upon the length of the insert. In addition, the expression of certain proteins from a gene inserted into recombinant PIV will result in attenuation of the virus due to the action of the protein. This has been described for IL-2 expressed in vaccinia virus (see, e.g.,
20   Flexner et al., _Nature_ 33:-259-62 (1987)) and also would be expected for gamma interferon.

        Deletions, insertions, substitutions and other mutations involving changes of whole viral genes or gene segments in recombinant PIV of the invention yield highly
25   stable vaccine candidates, which are particularly important in the case of immunosuppressed individuals. Many of these mutations will result in attenuation of resultant vaccine strains, whereas others will specify different types of desired phenotypic changes. For example, certain viral genes
30   are known which encode proteins that specifically interfere with host immunity (see, e.g., Kato et al., _EMBO. J._ 16:578-87 (1997), incorporated herein by reference). Ablation of such genes in vaccine viruses is expected to reduce virulence and pathogenesis and/or improve immunogenicity.
35   In preferred aspects of the invention, the modified PIV clones represent a chimera of two or more human PIV genomes, for example a clone containing polynucleotide sequences from HPIV3 joined to sequences from one or more

heterologous human PIV, such as HPIV1 and HPIV2. Thus,
individual genes or gene segments of human PIV3 may be
replaced or supplemented with counterpart genes or gene
segments from HPIV 1 or HPIV2, or visa versa. In one example
5   described hereinbelow, the invention provides a PIV clone,
rPIV3-1, into which both the HN and F glycoprotein genes of
HPIV1 are substituted for their counterpart genes in an HPIV3
background, yielding a chimeric virus having immunological
characteristics representative of both parental strains.

10   In additional aspects of the invention, chimeric PIV
or PIV clones having other alterations of genes or gene
segments, as described above, are further modified by
introducing one or more attenuating mutations adopted from a
biologically derived mutant PIV, e.g., HPIV3 JS *cp*45 to
15   achieve an attenuated, or further attenuated, chimeric mutant
derivative. For example, one or more human PIV coding or non-
coding polynucleotides may be substituted with a counterpart
sequence from a heterologous human PIV, bovine PIV or murine
PIV as described above, and this alteration may be combined
20   with one or more mutations specifying, e.g., a *ts*, *ca* or *att*
phenotype adopted from a biologically derived attenuated PIV
mutant, to yield an attenuated or further attenuated (i.e.,
compared to either the chimeric clone or biologically derived
parent virus) vaccine virus. Alternatively, functional
25   deletion of a non-essential gene or gene segment, such as the
C, D or V ORF, may be combined in a recombinant PIV with one
or more mutations specifying a *ts*, *ca* or *att* phenotype from
biologically derived PIV mutants to yield an attenuated
vaccine strain. These combinatorial modifications yield
30   recombinant PIV having desired phenotypic characteristics,
e.g., increased yield of virus, enhanced attenuation, and/or
genetic resistance to reversion from an attenuated phenotype,
due to the combined effects of the different selected
mutations.

35   In one combinatorial mutation design, a modified PIV
is provided which comprises a chimera of a human PIV genomic
or antigenomic sequence and at least one non-human PIV
sequence, for example a polynucleotide containing sequences

from both human and bovine PIV, and which also incorporates
one or more mutations adopted from biologically derived PIV,
e.g., one or more naturally occurring *ts*, *ca* or *att* mutations.
Alternatively, the modified PIV can be a chimera of two or

5   more human PIV genomes, for example a polynucleotide
containing sequences from HPIV3 joined to sequences from one
or more heterologous human PIVs, such as HPIV1 and HPIV2,
which further incorporates one or more *ts*, *ca att* or other
selected mutations from biologically derived PIV (e.g., a

10   nucleotide substitution specifying a *ts*, *ca* or *att* phenotype
adopted from a biologically derived mutant PIV strain such as
JS *cp*45). In more detailed aspects, individual genes or gene
segments of human PIV3 are replaced or supplemented with
counterpart genes or gene segments from HPIV1 or HPIV2, or

15   visa versa, in a clone that is attenuated or further
attenuated by, e.g., a nucleotide change encoding an amino
acid substitution conferring a *ts* mutation in the large
polymerase L gene. For example, the invention provides PIV
clones having the HN and/or F glycoprotein genes of HPIV1

20   substituted for their counterpart genes in an HPIV3
background, wherein the phenotype of the resultant chimeric
clone is further modified by *ts*, *ca* or *att* mutation(s) encoded
within one or more of the N, P, L, M, HN, F, C, D and V genes.
Various combinations from a menu of possible mutations

25   disclosed herein can be made to calibrate a vaccine virus to a
selected level of attenuation, immunogenicity and stability,
e.g., to achieve a satisfactorily attenuated and immunogenic,
chimeric virus having immunological characteristics
representative of multiple PIV strains. In one aspect,

30   recombinant PIVs are provided wherein at least one attenuating
mutation occurs in the PIV polymerase gene L (as exemplified
by the recombinants r942, r992, r1558, r942/992, r942/1558,
r992/1558, or r942/992/1558 described in the Examples below)
incorporated in a chimeric PIV background. For example,

35   useful chimeric PIV recombinants within this aspect of the
invention will have one or more genes or gene segments of the
HN and/or F glycoprotein genes from, e.g., HPIV1 substituted
for their counterpart gene(s) in a heterologous background,

41

e.g., in an HPIV3 clone, and will further incorporate one or more attenuating mutations, eg., nucleotide substitutions resulting in an amino acid change in the polymerase gene (such as change from Tyr to His at position 942, a change from Leu to Phe at position 992, and/or a change from Thr to Ile at position 1558) from a biologically derived PIV mutant. One such chimeric, attenuated recombinant exemplified hereinbelow is rPIV3-1.cp45L, a derivative of rPIV3-1 which incorporates all three L gene mutations specified above from JS cp45.

Yet additional mutations which can be incorporated in a chimeric PIV background for developing vaccine strains will be selected from biologically derived mutations in other genes, or will be created de novo in a recombinant genome by standard site directed mutagenesis or other purely recombinant mutagenic methods. Target genes for adopting biologically derived mutations or creating novel mutations in a recombinant PIV in this context include the nucleocapsid protein N, phosphoprotein P, large polymerase subunit L, matrix protein M, hemagglutinin-neuraminidase protein HN, fusion protein F and the C, D and V ORF products. Also targeted are extragenic sequences, eg., sequences in the 3' leader or trailer regions of a PIV genome. Exemplary mutations identified and incorporated in non-chimeric, recombinant PIV, described above will thus be readily incorporated within a chimeric PIV background, eg., as exemplified by rPIV3-1. These exemplary mutations include one or more mutations in the N protein, including specific mutations at a position corresponding to residues Val_{96} or Ser_{389} of JS cp45. In more detailed aspects, these mutations are represented as Val_{96} to Ala or Ser_{389} to Ala. Also desired for incorporation in chimeric PIV recombinants are mutations in the C protein, eg., a mutation at a position corresponding to Ile_{96} of JS cp45, preferably represented by a substitution of Ile_{96} to Thr, as described above. Further exemplary mutations for incorporation in a chimeric PIV background include one or more mutations in the F protein, for example adopted from JS cp45 at a position corresponding to residues Ile_{420} or Ala_{450}, eg., substitutions Ile_{420} to Val or Ala_{450} to Thr. Yet additional chimeric PIV

42

recombinants within the invention will adopt one or more amino acid substitutions in the HN protein, for example a mutation at a position corresponding to residue Val_{384} of JS cp45, such as Val_{384} to Ala. Yet additional chimeric recombinants will

5   incorporate one or more mutations in an extragenic sequence, eg., a 3' leader sequence of the recombinant genome or antigenome. Exemplary mutations in this context include mutations in the 3' leader occurs at one or more positions corresponding to nucleotide 23, 24, 28, and/or 45 of JS cp45,

10   for example a T to C change at nucleotide 23, a C to T change at nucleotide 24, a G to T change at nucleotide 28, or a T to A change at nucleotide 45. Yet additional extragenic mutations for incorporation within a chimeric PIV background include one or more mutations in a N gene start sequence, as

15   exemplified herein by a mutation in the N gene start sequence at a position corresponding to nucleotide 62 of JS cp45, such as a A to T change. These exemplary mutations evaluated and combined into recombinant PIV in the Examples below will be readily incorporated within a chimeric PIV recombinant using

20   the methods and tools provided herein, and will specify, individually and/or in combination, desired phenotypic changes to yield yet additional attenuated chimeric vaccine strains within the invention.

     In additional combinatorial mutation designs, modified

25   PIVs are provided which incorporate one or more of the foregoing ts, ca or att mutations adopted from biologically derived PIV or generated recombinantly in a PIV clone of the invention, in combination with another, distinct mutation disclosed herein (e.g., a deletion, addition, or rearrangement

30   of a PIV N, P, L, M, HN, F, C, D or V gene or gene segment, or a gene or gene segment from another source such as RSV or measles virus). Also in this case, various combinations from a menu of mutations disclosed herein can be made to calibrate the vaccine virus to a selected level of attenuation,

35   immunogenicity and stability. Thus, recombinant PIVs are provided which exhibit at least one attenuating mutation from a biologically derived PIV mutant, e.g., a mutation in the PIV polymerase gene L as found in JS cp45, or a recombinantly

43   -

generated mutation, and which further incorporates one or more
additional changes selected from, e.g., substitution or
introduction of a heterologous gene or gene segment from a
non-PIV source (e.g., an immunogenic RSV or measles gene or
5   epitope, or a gene encoding a cytokine), a change in the order
of viral genes to alter expression levels, removal of gene
overlap, substitution of a PIV genome promoter with its
antigenome counterpart, shortening, lengthening or removal of
intergenic regions, e.g., to increase capacity for inserting
10   foreign sequences, mutations in cis-acting signals to reduce
or increase RNA replication or transcription, insertion of
unique restriction sites, or deletion of even entire, non-
essential genes, among other changes.

For vaccine use, virus produced according to the
15   present invention can be used directly in vaccine
formulations, or lyophilized, as desired, using lyophilization
protocols well known to the artisan. Lyophilized virus will
typically be maintained at about 4°C. When ready for use the
lyophilized virus is reconstituted in a stabilizing solution,
20   e.g., saline or comprising SPG, Mg^{++} and HEPES, with or without
adjuvant, as further described below.

PIV vaccines of the invention contain as an active
ingredient an immunogenically effective amount of PIV produced
as described herein. The modified virus may be introduced
25   into a host with a physiologically acceptable carrier and/or
adjuvant. Useful carriers are well known in the art, and
include, e.g., water, buffered water, 0.4% saline, 0.3%
glycine, hyaluronic acid and the like. The resulting aqueous
solutions may be packaged for use as is, or lyophilized, the
30   lyophilized preparation being combined with a sterile solution
prior to administration, as mentioned above. The compositions
may contain pharmaceutically acceptable auxiliary substances
as required to approximate physiological conditions, such as
pH adjusting and buffering agents, tonicity adjusting agents,
35   wetting agents and the like, for example, sodium acetate,
sodium lactate, sodium chloride, potassium chloride, calcium
chloride, sorbitan monolaurate, triethanolamine oleate, and
the like. Acceptable adjuvants include incomplete Freund's

adjuvant, MPL™ (3-o-deacylated monophosphoryl lipid A; RIBI ImmunoChem Research, Inc., Hamilton, MT) and IL-12 (Genetics Institute, Cambridge MA), among many other suitable adjuvants well known in the art.

Upon immunization with a PIV composition as described herein, via aerosol, droplet, oral, topical or other route, the immune system of the host responds to the vaccine by producing antibodies specific for PIV virus proteins, e.g., F and HN glycoproteins. As a result of the vaccination with an immunogenically effective amount of PIV produced as described herein, the host becomes at least partially or completely immune to PIV infection, or resistant to developing moderate or severe PIV infection, particularly of the lower respiratory tract.

The host to which the vaccines are administered can be any mammal which is susceptible to infection by PIV or a closely related virus and which host is capable of generating a protective immune response to the antigens of the vaccinizing strain. Accordingly, the invention provides methods for creating vaccines for a variety of human and veterinary uses.

The vaccine compositions containing the PIV of the invention are administered to a host susceptible to or otherwise at risk for PIV infection to enhance the host's own immune response capabilities. Such an amount is defined to be a "immunogenically effective dose." In this use, the precise amount of PIV to be administered within an effective dose will depend on the host's state of health and weight, the mode of administration, the nature of the formulation, etc., but will generally range from about 10^3 to about 10^6 plaque forming units (PFU) or more of virus per host, more commonly from about 10^4 to 10^5 PFU virus per host. In any event, the vaccine formulations should provide a quantity of modified PIV of the invention sufficient to effectively protect the host patient against serious or life-threatening PIV infection.

The PIV produced in accordance with the present invention can be combined with viruses of other PIV serotypes or strains to achieve protection against multiple PIV

45   −

serotypes or strains. Alternatively, protection against
multiple PIV serotypes or strains can be achieved by combining
protective epitopes of multiple serotypes or strains
engineered into one virus, as described herein. Typically

5   when different viruses are administered they will be in
admixture and administered simultaneously, but they may also
be administered separately. Immunization with one strain may
protect against different strains of the same or different
serotype.

10   In some instances it may be desirable to combine the
PIV vaccines of the invention with vaccines which induce
protective responses to other agents, particularly other
childhood viruses. In another aspect of the invention the PIV
can be employed as a vector for protective antigens of other

15   pathogens, such as respiratory syncytial virus (RSV) or
measles virus, by incorporating the sequences encoding those
protective antigens into the PIV genome or antigenome which is
used to produce infectious PIV, as described herein. The
cloning of RSV cDNA and other disclosure relevant to the

20   invention is described in copending United States patent
applications Serial Nos. 08/534,768, 60/021,773, 08/720,132,
60/046,141, 60/047,634, and 08/892,403, and PCT patent
application PCT/US97/12269, each incorporated herein by
reference.

25   Single or multiple administrations of the vaccine
compositions of the invention can be carried out. In neonates
and infants, multiple administration may be required to elicit
sufficient levels of immunity. Administration should begin
within the first month of life, and at intervals throughout

30   childhood, such as at two months, six months, one year and two
years, as necessary to maintain sufficient levels of
protection against native (wild-type) PIV and/or other subject
viral infections. Similarly, adults who are particularly
susceptible to repeated or serious PIV infection, for example

35   health care workers, day care workers, family members of young
children, the elderly, and individuals with compromised
cardiopulmonary function, may require multiple immunizations
to establish and/or maintain protective immune responses.

Levels of induced immunity provided by the vaccines
of the invention can be monitored by measuring amounts of
neutralizing secretory and serum antibodies. Based on these
measurements, vaccine dosages can be adjusted or vaccinations

5   repeated as necessary to maintain desired levels of
protection. Further, different vaccine viruses may be
advantageous for different recipient groups. For example, an
engineered PIV strain expressing an additional protein rich in
T cell epitopes may be particularly advantageous for adults

10   rather than for infants.

In yet another aspect of the invention the PIV is
employed as a vector for transient gene therapy of the
respiratory tract. According to this embodiment the
recombinant PIV genome or antigenome incorporates a sequence

15   which is capable of encoding a gene product of interest. The
gene product of interest is under control of the same or a
different promoter from that which controls PIV expression.
The infectious PIV produced by coexpressing the recombinant
PIV genome or antigenome with the N, P, L and other desired

20   PIV proteins, and containing a sequence encoding the gene
product of interest, is administered to a patient.
Administration is typically by aerosol, nebulizer, or other
topical application to the respiratory tract of the patient
being treated. Recombinant PIV is administered in an amount

25   sufficient to result in the expression of therapeutic or
prophylactic levels of the desired gene product.
Representative gene products which may be administered within
this method are preferably suitable for transient expression,
including, for example, interleukin-2, interleukin-4, gamma-

30   interferon, GM-CSF, G-CSF, erythropoietin, and other
cytokines, glucocerebrosidase, phenylalanine hydroxylase,
cystic fibrosis transmembrane conductance regulator (CFTR),
hypoxanthine-guanine phosphoribosyl transferase, cytotoxins,
tumor suppressor genes, antisense RNAs, and vaccine antigens.

35

The following examples are provided by way of
illustration, not limitation.

EXAMPLE I
Construction of Plasmid p218(131)
Encoding Negative sense PIV Genomic RNA

5   A full cDNA clone designated p218(131) (Fig. 1; SEQ
ID NO: 1) (deposited under the terms of the Budapest Treaty
with the American Type Culture Collection (ATCC) of 12301
Parklawn Drive, Rockville, Maryland 20852, U.S.A., and granted
the designation 97991) was constructed to encode the complete
10   15462 nt genomic sequence of HPIV3 JS strain. A hepatitis
delta ribozyme was placed abutting the 3ʹ end of the genomic
sequence such that self-cleavage would yield the 3ʹ end of
HPIV3 (Perrotta and Been, Nature 350: 434-436, (1991),
incorporated herein by reference in its entirety). A T7
15   transcription terminator was placed following the delta
ribozyme. The T7 promoter was placed adjacent to the 5ʹ end
of the genomic sequence such that the 5ʹ terminal nucleotide
of the HPIV3 genome was the first nucleotide synthesized. In
this configuration, the cDNA encodes a complete negative-sense
20   copy of PIV3 genomic RNA containing the correct genomic
termini without any additional heterologous nucleotides.
          The HPIV3 cDNA was assembled from 14 overlapping
subclones (termed A*-L, which letters in parentheses designate
individual plasmids and do not refer to specific viral genes)
25   constructed by reverse transcription (RT) and polymerase chain
reaction (PCR) of RNA isolated from virions purified by
sucrose gradient centrifugation (Stokes et al., supra, 1992;
Stokes et al., supra, 1993, each incorporated herein by
reference in its entirety). The subclones spanned the
30   following nucleotides of genomic RNA (numbered with the 3ʹend
designated as position 1): 1-2058 (A*), 1874-3111 (Aʹ),
3086-5140 (C), 4348-5276 (Cʹ), 5072-6695 (D*), 5904-8532 (E),
7806-9898 (F), 9632-10740 (Fʹ), 9760-10955 (G), 10862-11925
(H), 11835-12868 (I), 12426-13677 (J), 13630-14496 (K), and
35   14467-15462 (L). Each fragment was cloned into pBluescript
KSII (Strategene, La Jolla, CA) using conventional cloning
techniques and was sequenced completely.

Plasmid p(L) was then subjected to site-directed
mutagenesis to introduce the T7 promoter via a single-stranded
DNA intermediate according to the MUTA-GENE procedure (BioRad,
Hercules, CA). The T7 promoter was positioned so that
5   transcription initiates at the precise 5´ end of the HPIV3
genome using the negative-sense mutagenic primer:
5´-*AATACGACTCACTAT*A*ACCAAACAAGAGAAG*-3´ (SEQ ID NO: 2; T7
sequences are italicized, HPIV3-specific sequences are
underlined, and the 5´-end HPIV3 nucleotide, genome position
10   15462, is indicated by an asterisk). This modified p(L) was
designated p(L*). Plasmid p(E) was modified to yield p(E*) by
the same method using the negative-sense mutagenic
oligonucleotide 5´-CCAAGTACTATGAGATGCTTGATT-3´ (SEQ ID NO: 3)
to insert three nucleotide substitutions (underlined) into the
15   HN gene at HPIV3 position 7903, 7913, 7915 (Fig. 1). These
substitutions removed an *Hga* I site, inserted a *Sca* I site,
and modified amino acid 370 of the encoded HN protein such
that the epitope recognized by monoclonal antibodies (mAb)
423/6 and 170/7 was ablated (van Wyke Coelingh et al., J.
20   Virol. 61:1473-1477, (1987), incorporated herein by
reference). The p(E*) to p(K) subclones were assembled in a
step-wise fashion into the p(L*) plasmid to give
p(E*FF´GHIJKL*) (Fig. 1A). This plasmid includes HPIV3
nucleotides 5904-15462 with the T7 promoter adjacent to
25   nucleotide 15462 at the 5´ end of the genome. Subclones p(A*)
to p(E) were assembled into a second, overlapping subclone,
p(A*A´CC´D*E) which contained HPIV3 nucleotides 1-8532.

Both subclones p(E*FF´GHIJKL*) and p(A*A´CC´D*E)
were sequenced completely. In addition to the introduced
30   point mutations described above, the cDNA differed from the
authentic JS HPIV3 sequence (Stokes et al., supra, 1992) by a
single nucleotide substitution at position 1615 which was
within the N gene and caused a substitution at amino acid 506
in the encoded protein. Three other nucleotide substitutions
35   were found at positions 10355, 11333, and 15248 in the L gene
which did not change the encoded protein (Fig. 2). These
three noncoding changes were retained as additional sequence
markers to identify recombinant virus (designated rPIV)

49

derived from cDNA, and the mutation in the N gene was
corrected as described later.

Subclone p(A*A´CC´D*E) was then modified to insert
the hepatitis delta virus ribozyme and T7 terminator adjacent
to HPIV3 position 1. An HPIV3 minigenome in which the 3´ end
of the HPIV3 genome (<u>GGT</u>↓**GGG**) (underlined) was generated
through self-cleavage of a flanking hepatitis delta virus
antigenomic ribozyme (shown in part in bold-type) was
previously constructed (Dimock and Collins, <u>J. Virol.</u> <u>67</u>:
2772-2778, (1993); Perrotta and Been, <u>supra</u>, (1991), each
incorporated herein by reference in its entirety). The
ribozyme in turn was followed by a T7 transcription
terminator. This minigenome cDNA was used as a template in a
PCR reaction which modified the sequence adjacent to the
ribozyme cleavage site to be a *Sma* I site (CCC↓GGG) and placed
an *Apa*I site (GGGCC↓C) on the downstream side of the T7
terminator. The PCR product was cloned into pKSII which had
been digested with *Bss*HII and made blunt-ended by filling in,
yielding p218.

        p218 was designed such that any sequence could be
introduced into the opened *Sma* I site by blunt-end ligation
and its RNA transcript would be cleaved at the delta ribozyme
cut site (NNN↓GGG). The p(A*A´CC´D*E) subclone was digested
with *Hga* I and *Sal* I (8533), which released the HPIV3 cDNA,
and was filled in with dNTPs and T4 DNA polymerase to give
blunt termini. The *Hga* I site is 10 nucleotides upstream of
HPIV3 position 1 and, when digested and filled in, leaves a
blunt terminus beginning with HPIV3 position 1. The modified
Hga I-*Sal* I fragment was gel purified and cloned into the *Sma*
I site of p218. The mutation in the N gene (T at nt 1615) was
corrected to the JS wt sequence (A at nt 1615) (see GenBank
accession #Z11575, incorporated herein by reference) using
Kunkel mutagenesis (Kunkel et al., <u>Methods Enzymol.</u> <u>154</u>: 367-
382, (1987), incorporated herein by reference in its
entirety). This plasmid was designated p218(A*A´CC´D*E) (Fig.
1).

The *Xho* I-*Ngo* MI fragment of p(E*FF´GHIJKL*), which contained the T7 promoter and the HPIV3 cDNA from nucleotides 7438-15462, was cloned into the *Xho* I-*Ngo* MI window of p218(A*A´CC´D*E) (Fig. 1). This joined the two fragments of

5   HPIV3 cDNA at HPIV3 nucleotide 7438, yielding a plasmid containing a full-length HPIV3 cDNA encoding a negative-sense genomic RNA with the above-mentioned three desired mutations in the HN gene and three incidental mutations in the L gene. The final construct, designated p218(131) (Fig. 1; SEQ ID NO:

10   1), was then sequenced in its entirety by automated sequencing at the NCI Frederick Cancer Research and Development Center (Frederick, MD) using the Taq DYE Deoxy Terminator cycle sequencing kit (ABI, Foster City, CA). This identified an additional change in the HN gene, namely a change of C to T in

15   the HN gene at position 7593 which changed HN amino acid 263 from threonine to isoleucine that is also indicated in Fig. 2.

EXAMPLE II

20   <u>Transcription and RNA Replication System for HPIV3</u>

The present example describes compositions and methods for producing a reconstituted transcription and RNA replication system for human parainfluenza virus type 3

25   (HPIV3). This exemplary system was developed using components expressed intracellularly from transfected plasmids driven by a T7 RNA polymerase supplied by a vaccinia virus recombinant. The system is based on a negative-sense analog of HPIV3 genomic RNA in which the viral genes were deleted and replaced

30   with a polynucleotide encoding bacterial chloramphenicol acetyl transferase (CAT). The N, P and L proteins are expressed from cotransfected plasmids so as to direct efficient transcription and RNA replication. Transcription according to this example yields subgenomic polyadenylated

35   mRNA, which can be readily isolated, e.g., by oligo(dT) chromatography. RNA replication according to this example yields mini-antigenome and progeny minigenome, which are shown

to be encapsidated based on resistance to digestion with
micrococcal nuclease.

A)   Viruses and cells

A vaccinia virus recombinant, vTF7-3, that expresses
bacteriophage T7 RNA polymerase, was provided as described by
Fuerst et al. (Proc. Natl. Acad. Sci. U.S.A. 83: 8122-8126,
1986, incorporated herein by reference in its entirety).
HEp-2 monolayer cultures were maintained at 37°C in 5% CO_2
with OptiMEM 1 (Life Technologies, Gaithersburg, MD)
supplemented with 2% fetal bovine serum (FBS), 50 µg/ml
gentamicin sulfate and 2mM glutamine.

B)   cDNAs

cDNAs corresponding to ORFs of the N, P, and L genes
of the JS strain of HPIV3 (GenBank #Z11575; Stokes et al.,
1992) were individually cloned into the Nco I-Sal I window of
plasmid pTM-1, in which transcription is mediated by T7 RNA
polymerase and translation by an internal ribosome entry site
preceding the foreign ORF (Elroy-Stein et al., Proc. Natl.
Acad. Sci. U.S.A. 86: 6126-6130 (1989), incorporated herein by
reference in its entirety). Each gene was first modified by
polymerase chain reaction (PCR) to place an Nco I or Nco
I-compatible site at the translational start site and a Sal I
site on the downstream end.

The plasmid p(131), which is similar to p218(131)
except that it lacks the hepatitis delta virus ribozyme, was
used as a template for each PCR. The primers used to amplify
the N ORF were CCCTATAATTTCAACATGTTGAGCCTATTTG (SEQ ID NO: 4;
forward primer relative to positive-sense) and
GATTAAAATGTTGGTCGACTTAGTTGCTTCC (SEQ ID NO: 5; italics
represent restriction enzyme sites, and the translational
start site is in bold). The PCR product, a 1578 bp fragment
flanked by an Afl III and Sal I site, was cloned into the Nco
I- Sal I window of pTM-1 to yield pTM(N).

The primers used to amplify the PIV3 phosphoprotein
(P) ORF were 5-'CCATAGAGAGTCCATGGAAAGCGATGCTAAAAACTATC-3' (SEQ
ID NO:6; forward primer) and 5'-
CGGTGTCGTTTCTTTGTCGACTCATTGGCAATTGTTG-3' (SEQ ID NO:7; reverse
primer). A full-length cDNA of JS strain of genomic RNA

52

(p131) was used as template for the PCR. The resultant PCR product was an 1851 bp fragment flanked by an *Nco* I and *Sal* I restriction site (in italics). The PCR product was then cloned into the *Nco* I-*Sal* I window of pTM-1 to yield pTM(P).

5   A second PCR was performed to amplify the PIV3 phosphoprotein P ORF without the C ORF. p131 was again used as template cDNA. A different forward primer and the same reverse primer were used to amplify the PIV3 P ORF without C; 5'-CCATAGAGAGT*CCATGG*AAAGCGA<u>C</u>GCTAAAAACTATC-3' (SEQ ID NO: 74;

10   forward primer) and 5'-CGGTGTCGTTTCTTT*GTCGAC*TCATTGGCAATTGTTG-3' (SEQ ID NO:7; reverse primer). The resultant PCR product was an 1851 bp fragment flanked by an *Nco* I and *Sal* I restriction site (designated by italics). The underlined nucleotide in the forward primer represents a nucleotide

15   substitution which is silent in the P ORF but changes the start codon of the C ORF to threonine. The next start codon for the C ORF is more than 400 nucleotides downstream. Thus, only the P protein would be produced. The PCR product was then cloned into the *Nco* I-*Sal* I window of pTM-1 to yield a

20   second plasmid, pTM(P no C).

        The L ORF of HPIV3 was cloned into pTM-1 in three parts: the ends were derived from PCR products and the main body was a restriction fragment from p218(131). The upstream end of the L ORF was amplified using the primers

25   GCAAAGCGTG*CCCGGGCCATGG*ACACTGAATCTAACAATGGC (SEQ ID NO: 8) and GAAATTCCTTA*ATCGAT*TCTCTAGATTC (SEQ ID NO: 9). This yielded the 1,020-bp PCR product L1 in which positions 8625-9645 of the full-length genome were flanked by *Sma* I and *Nco* I sites on the upstream end and a *Cla* I site on the downstream end (all

30   three sites are italicized). The downstream end of the L ORF was amplified using the primers CCCATCAACTGTAACA*TACGTA*AGAAAGAC (SEQ ID NO: 10) and GGTTAGGATAT*GTCGAC*ATTGTATTTATG (SEQ ID NO: 11). This yielded the 1,733-bp PCR product L2 in which positions 13,645-15,378 of the full-length genome were flanked

35   by a *SnaB* I and *Sal* I site (italicized). Plasmid p(131) was digested with *Cla* I and *Pst* I to yield the 4,487-bp fragment L middle containing positions 9,630-14,120 of the full-length genome. L1 and L middle were joined at the common *Cla* I site

and cloned into the *Sma I-Pst I* window of pBluescript to yield
p(Ll+L middle). The L2 fragment was then cloned into the *Pst
I-Sal I* window of p(Ll+L middle) to yield the complete L ORF
flanked by *Nco I* and *Sal I*. This was then cloned into the *Nco
I-Sal I* window of pTM-1 to yield pTM(L). The sequences of
PCR-generated regions of pTM(N) (SEQ ID NO: 20), pTM(P) (SEQ
ID NO: 21), and pTM(L) (SEQ ID NO: 22) were confirmed by the
dideoxynucleotide sequencing method.

To increase the efficiency of T7 transcription,
certain modifications were made to a cDNA construct encoding a
negative-sense PIV minigenome, called PIV3-CAT(-) (Dimock and
Collins, *J. Virol.* 67: 2772-2778 (1993), incorporated herein
by reference in its entirety). PIV3-CAT(-) includes the
3´-terminal 111 nucleotides and 5´-terminal 115 nucleotides of
the HPIV3 genome fused to a negative-sense copy of the CAT
ORF. This cDNA was designed to yield, upon linearization with
HgaI and transcription with T7 RNA polymerase, a minigenome
containing the exact correct ends of the HPIV3 genome. Two
successive rounds of PCR, using mutagenic oligonucleotides
which added successive extensions to the cDNA end, were used
to replace the *HgaI* site with the hepatitis delta ribozyme
(Perotta and Been, *Nature* 350: 434-436 (1991), incorporated
herein by reference in its entirety), such that self-cleavage
generates the correct 3´ HPIV3 genomic end. A T7
transcriptional termination signal was inserted immediately
after the ribozyme (Fig. 3) to yield PIV3-CAT-delta.

PIV3-CAT-delta cDNA was modified by PCR mutagenesis
to insert one, two or three G residues between the T7 promoter
and the 5´ end of the minigenome, using restriction sites
flanking the trailer and T7 promoter. This modification
yielded increased efficiency of T7 transcription. In
preliminary experiments, the minigenome containing two G
residues, called PIV3-CAT-GG, was the most active in the
expression of CAT in the reconstituted transcription and
replication system described below, and was used for all
subsequent derivatives.

The PIV3-CAT-GG cDNA was further modified by
overlapping PCR mutagenesis to introduce modifications

simultaneously at two sites, as follows. First, the sequence
T_7CT, which contains tandem transcriptional termination motifs
for the vaccinia virus early-stage RNA polymerase (T_5NT) (Yuen
and Moss, Proc. Natl. Acad. Sci. U.S.A. 84: 6417-6421 (1987),

5   incorporated herein by reference in its entirety), was
inserted into the positive-sense strand cDNA strand between
the delta ribozyme and T7 transcriptional terminator (Fig. 1).
This motif, and a second motif described herein below, were
added to prevent promiscuous transcription of the CAT gene by

10   the vaccinia virus early RNA polymerase. Second, the
minigenome cDNA insert was modified at the junction between
the N nontranslated region and CAT gene to contain (i) the
insertion of a second vaccinia virus termination motif (T_5AT)
into the positive-sense cDNA strand at positions 103 to 109

15   relative to the encoded minigenome, and (ii) the insertion of
0 to 6 G residues (negative-sense) at minigenome position 112
(Fig. 3). The overlapping PCR mutagenesis involved three sets
of reactions (PCR 1, 2 and 3) performed as follows. PCR 1 was
a set of seven parallel reactions [PCR1 (0) to (+6)] which

20   used the PIV3-CAT-GG cDNA as a template and the following two
mutagenic oligonucleotides as primers: the forward primer was:
$^{-111}$GGGGTTATGCTACTGCAGGC**TTTTTTTTCT**CCCTTAGCCATCCG^{-62} (SEQ ID NO:
12) and the reverse primer was:
^{124}CTC*CATTCTAGA*(N)**TTATAAAAA**TTATAGAGTTCCC^{90} (SEQ ID NO: 13). The

25   bold sequence in the first oligonucleotide is the upstream
tandem vaccinia terminator, and the bold sequence in the
second oligonucleotide is the second terminator. This
reaction amplified the ribozyme and adjacent leader region and
inserted the mutations described above. PCR 2 was a single

30   reaction that used the PIV3-CAT-GG cDNA as a template, and a
forward primer that hybridized in plasmid sequence upstream of
a unique NgoMI site (Fig. 3), and a reverse primer
complementary to the forward primer of reaction one. Thus,
the products of PCR 1 and 2 overlapped at this latter

35   sequence. The products of PCR 1 (0) to (+6) and PCR 2 were
gel purified. The products of PCR1 (0) to (+6) were each
mixed separately with an aliquot of PCR 2 product and
amplified in a third reaction (PCR3 (0) to (+6)) which also

contained the forward primer of PCR 2 and the reverse primer
of PCR 1. The products of PCR3 (0) to (+6) were digested with
NgoMI, which cuts in plasmid-specific sequence, and XbaI,
which cuts at the upstream end of the CAT gene (Fig. 3), and
5   cloned into the NgoMI-XbaI window of PIV3-CAT-GG. This
resulted in a panel of cDNAs encoding minigenomes which were
named according to the number of inserted G residues: PIV3-CAT
0 to PIV3-CAT +6. The structures of all DNA regions derived
from PCR were confirmed by dideoxynucleotide sequencing.

10   C)   Transfection

             HEp-2 cells were grown to 90% confluence in 6 well
plates. Each well of a six-well plate (1.5 X 10^6 cells) was
transfected with 0.4 μg pTM(P), 0.4 μg pTM(N), 0.05 μg pTM(L),
and 0.4 μg minigenome plasmid. The plasmids were added to 0.1
15   ml of OptiMEM (Life Technologies) and mixed with 0.1 ml of
OptiMEM containing 12 μl of LipofectACE (Life Technologies).
After an incubation period of approximately 15 minutes at room
temperature, 0.8 ml of OptiMEM 1 containing 2% calf serum and
1.5 X 10^7 pfu of vTF7-3 was added to each well. The plates
20   were incubated at 37°C for 12 hours after which the media was
replaced with fresh OptiMEM 1 containing 2% fetal bovine
serum. The cells were then incubated at 37°C for a total of
48 hours and harvested for RNA analysis and CAT assay. Each
minigenome was represented in triplicate (3 wells) which was
25   scraped into the medium and pooled.

             D)   CAT assay

             An aliquot representing 3.33% (1.5 X 10^5 cells) of
each pooled sample of harvested cells described above was
removed for CAT assay. The aliquot was centrifuged at 1,000
30   rpm for 5 minutes and the supernatant discarded. The cell
suspension was washed with 1 ml of 40 mM Tris, pH 7.5, 1 mM
EDTA, 150 mM NaCl and resuspended in 50 μl 0.25 M Tris, pH
7.5. Lysate was prepared by three cycles of freezing and
thawing and clarified by centrifuging at 8,000 rpm for 5
35   minutes. 1 μl of lysate was assayed for the ability to
acetylate D-threo-[dichloroacetyl 1-^{14}C]chloramphenicol
(Amersham) using a conventional assay (Gorman et al., Mol.
Cell. Biol. 2: 1044-1051 (1982), incorporated herein by

reference in its entirety). Acetylation was visualized by
thin-layer chromatography and quantified by phosphoimager
analysis (Molecular Dynamics, Sunnyvale, CA).

E)   RNA analysis

5   The remaining cell harvest of each pooled sample was
divided into three equal parts for isolation of encapsidated
RNA, total RNA, and mRNA. The three aliquots were centrifuged
at 1,000 rpm for five minutes and the supernatants discarded.
Two aliquots of cell suspension were resuspended in 50 μl of

10   RSB (10mM NaCl, 10mM Tris, pH 7.5, 1.5mM MgCl$_2$) containing 1%
Triton X-100, 0.5% DOC. 50 μl of 10 mM Tris 7.5, 1 mM CaCl$_2$,
and 20 μg (1mg/ml stock) of micrococcal nuclease was then
added to one aliquot, and the other received the same mixture
without micrococcal nuclease (Baker & Moyer, J. Virol. 62:

15   834-838 (1988), incorporated herein by reference in its
entirety). The purpose of the micrococcal nuclease was to
destroy nonencapsidated RNA, and the conditions used had been
optimized in preliminary experiments. The mixtures were
incubated at 30°C for 30 min and the RNA was isolated with

20   Trizol (Life Technologies) according to the procedure of the
supplier. The third aliquot of cell suspension was processed
for RNA purification with Trizol and the purified RNA was
separated by oligo(dT) cellulose chromatography into
polyadenylated and nonpolyadenylated fractions (Grosfeld et

25   al., J. Virol. 69: 5677-5686 (1995), incorporated herein by
reference in its entirety). RNA samples were run on 1.5%
agarose gels containing 0.44 M formaldehyde, transferred to
nitrocellulose (Chomczynski, Anal. Biochem. 201: 134-139
(1992), incorporated herein by reference in its entirety),

30   hybridized with strand specific riboprobes, and quantified by
phosphoimager analysis.

EXAMPLE III

Construction and Expression of Modified PIV3 Minigenomes

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           In the present example, a panel of cDNAs was
constructed to encode PIV3 minigenomes which differed in
length by single nucleotide increments. Transcription and RNA

replication in this reconstituted system were the most
efficient for the minigenome whose length was an even multiple
of six. In this context, members of the *Paramyxovirus* and
Morbillivirus genera typically abide by a "rule of six," i.e.,
5   genomes (or minigenomes) replicate efficiently only when their
nucleotide length is a multiple of six (thought to be a
requirement for precise spacing of nucleotide residues
relative to encapsidating NP protein). However, the present
findings illustrate that minigenomes whose lengths were one
10   nucleotide greater than or less than an even multiple of six
were surprisingly active, especially in RNA replication.
        A panel of seven cDNAs was constructed to encode
seven PIV3-CAT minigenomes, called PIV3-CAT 0 to +6, that
differ in length by single-nucleotide increments (Fig. 3).
15   Each minigenome is a short negative-sense analog of HPIV3
genomic RNA in which the viral genes had been deleted and
replaced with a negative-sense copy of the CAT ORF. The CAT
ORF is flanked by nontranslated segments of the N and L genes,
the N GS and L GE transcription motifs, and the 3´ leader and
20   5´ trailer extragenic termini of genomic RNA. The 5´ end of
each PIV3-CAT minigenome is defined by the adjacent promoter
for T7 RNA polymerase. This promoter was constructed to
contribute an extension of two nonviral G residues to the 5´
end of the encoded minigenome, as described above. The
25   presence of additional G residues adjacent to the
nontranscribed core of the T7 promoter improves its
transcriptional efficiency, and preliminary work showed that
presence of two G residues provided the highest levels of
activity in the reconstituted system described below. These
30   two G residues are not included in minigenome length
calculations. The 3´ minigenome end is created by
self-cleavage by an abutting hepatitis delta virus ribozyme,
which would generate the correct 3´-terminal nucleotide. The
seven PIV3-CAT minigenomes differ by single-nucleotide
35   increases in length due to the insertion of 0 to 6 G residues
at the junction between the N nontranslated region and the CAT
gene (Fig. 3), and range in length from 898 to 904
nucleotides. The PIV3-CAT +2 minigenome is the only one which

58

is an even multiple of six (it should be noted that each
minigenome contained two additional nonviral G residues at the
5' end contributed by the T7 promoter, but it is assumed that
these would be lost during intracellular HPIV3-mediated RNA

5   replication).

Each PIV3-CAT cDNA was transfected into HEp-2 cells
that had been infected with vTF7-3, a vaccinia virus
recombinant that expresses T7 RNA polymerase. Plasmids
encoding the N, P and L proteins under the control of the T7

10   promoter were transfected in parallel. The P cDNA had been
modified by site-directed mutagenesis to eliminate the
translational start site of the C ORF, as described above.
Cells were harvested at 48 h post-infection. An aliquot of the
cell suspension was processed for CAT enzyme assay (Fig. 4).

15   The remaining cells were divided into three equal aliquots and
processed for RNA analysis as described below.

The minigenome cDNA was further modified to contain
two tandem vaccinia virus early-gene transcription termination
motifs (T₇NT) in the positive-sense plasmid strand upstream of

20   the PIV3-CAT insert, and a third one (T₅AT) in the same strand
immediately upstream of the CAT ORF (Fig. 3). These were
designed to minimize promiscuous transcription of the CAT ORF
by vaccinia virus polymerase (Grosfeld et al. (1995), supra).
CAT expression was reproducibly detected when each of PIV3-CAT

25   minigenomes was complemented by the N, P and L plasmids (Fig.
4), and detection of CAT was dependent on all three PIV3
proteins. However, expression was much higher for PIV3-CAT+2,
which has a nucleotide length that is an even multiple of six.
Preferred ratios and amounts of the minigenome and support

30   plasmids were determined based on CAT enzyme expression.

EXAMPLE IV
Synthesis of Positive-Sense RNAs by PIV Minigenomes

35   Transcription and replication of PIV minigenomes was
confirmed by detection of RNA products of both processes. As
described in the foregoing Example, three equal aliquots of
cell suspension were taken for RNA analysis. One aliquot was

used for oligo(dT) analysis, as described below. The other
two aliquots were lysed with detergent and incubated with
micrococcal nuclease or mock-treated. RNA was then isolated,
separated by electrophoresis on formaldehyde agarose gels,
transferred to nitrocellulose, and analyzed by hybridization
with negative-sense CAT riboprobe. RNA from
micrococcal-treated and mock-treated lysates are shown in Fig.
5A upper and lower panels, respectively.

        Analysis of RNA from mock-treated lysates showed
that complementation of each minigenome with the N, P and L
plasmids resulted in the synthesis of a band of RNA which was
very similar in size to a marker consisting of RNA expressed
by the 931-nucleotide RSV-CAT C2 minigenome (Grosfeld et al.
(1995), supra). Phosphorimagery analysis is shown in Fig. 5B.
Little or no RNA was detected when the N or L plasmids were
omitted, confirming that these RNAs are products of the
reconstituted PIV3 polymerase.

        Each PIV3-CAT minigenome is expected to encode two
positive-sense RNAs, namely the mini-antigenome and the
subgenomic CAT mRNA. Each mini-antigenome is expected to be
the exact complement of its minigenome, which was 898 to 904
nucleotides in length. The predicted subgenomic mRNA is
defined by the GS and GE signals, and is expected to be 804
nucleotides in length and contain a polyA tail of 100 to 200
nucleotides.

        Detection of a single gel band of positive-sense RNA
in Fig. 5A (lower panel) suggested that the antigenome and
mRNA were not resolved by gel electrophoresis. Accordingly,
treatment with micrococcal nuclease was used to identify
antigenome RNA, since the antigenome (and genome) but not mRNA
would be encapsidated and resistant to digestion. The use of
micrococcal nuclease for this purpose is well established
(Baker & Moyer (1988), supra), and the conditions selected
were verified with RSV minireplicons and shown to completely
degrade mRNA contained in the HEp-2 cell lysates. Residual
RNA was purified and analyzed by Northern blot analysis with
negative-sense riboprobe (Fig. 5A, upper panel) and
quantitated by phosphorimagery (Fig. 5B; note that in this

analysis the micrococcal-treated and untreated RNA amounts were normalized separately). These investigations revealed the presence of a population of protected RNA corresponding to the positive-sense encapsidated mini-antigenome. Among

5   several experiments, this protected RNA accounted for approximately 3 to 15% of the positive-sense RNA.

For both the total and the micrococcal-resistant RNA, accumulation was greatest in the case of the +2 minigenome, which is 900 nucleotides in length and thus a

10   multiple of six. However, substantial amounts of RNA also accumulated in the case of the minigenomes which did not exhibit a length corresponding to a multiple of six nucleotides, in particular minigenomes +1 and +3 which were one nucleotide longer or shorter than the +2 minigenome. In

15   fact, the amount of encapsidated antigenome produced by the +1 and +3 mingenomes was 85% and 72% that of the +2 minigenome (Fig. 5B). Even the least efficient minigenome, the +5 minigenome, was 20% as active as the +2 minigenome as determined by measurement of accumulated encapsidated RNA. In

20   the case of measurements to detect total positive-sense RNA, the +1 and +3 minigenomes produced 52% and 45% as much total RNA as the +2 minigenome.

To confirm the presence of subgenomic mRNA, the final aliquot of harvested cell suspension was processed for

25   RNA purification. The RNA was then subjected to oligo(dT) chromatography. RNAs which failed to bind, and those which bound and were eluted in low salt buffer, were analyzed by Northern blot hybridization (Fig. 6A) and phosphorimagery (Fig. 6B; note that in this case the bound and unbound are

30   normalized together relative to the bound RNA of the +2 minigenome). These assays showed that approximately 64% of positive-sense RNA was polyadenylated, as expected for subgenomic mRNA. The accumulation of mRNA was greatest for the +2 minigenome. However, substantial amounts of mRNA also

35   were observed for the other minigenomes. The amount of mRNA synthesized by the +1 and +3 minigenomes was 30% and 20% respectively compared to that synthesized by the +2

minigenome, and was approximately 13% for the least active
minigenomes.

EXAMPLE V

5   Synthesis of Negative Sense RNA by PIV Minigenomes

The various PIV3-CAT minigenomes described in the
foregoing examples directed synthesis of mRNA and
positive-sense encapsidated mini-antigenome, the latter
10   representing the first step in RNA replication. The second
step in RNA replication involves synthesis of encapsidated
progeny minigenome from the mini-antigenome product. To
evaluate this latter process, the samples of RNA from
mock-treated and nuclease-treated lysates described in the
15   preceding Example were analyzed by Northern blot hybridization
with positive-sense CAT riboprobe (Fig. 7A) and quantitated by
phosphorimagery (Fig. 7B).
Analysis of RNA from mock-treated lysates (Fig. 7A,
lower panel) showed that considerable amounts of minigenome
20   accumulated intracellularly in all samples, including negative
controls in which the N or L support plasmid was omitted. The
analyses described in Figs. 5A-B and 6A-B showed that the
synthesis of positive-sense RNA was insignificant under these
conditions. Therefore, the minigenome observed in the absence
25   of N or L could not be the product of RNA replication mediated
by the reconstituted HPIV3 polymerase, and instead must be the
product of T7 transcription of transfected plasmid.
Minigenome produced by the reconstituted HPIV3
polymerase is expected to be encapsidated, whereas much of the
30   minigenome produced by T7 RNA polymerase is expected to be
unencapsidated. Therefore, RNA from the same micrococcal
nuclease-treated samples described for Figs. 5A-B were used to
prepare a second blot, which was hybridized with
positive-sense CAT riboprobe (Fig. 7A, upper panel). This
35   showed that all minigenome RNA accumulated in the absence of
the N protein was degraded (Fig. 7A, upper panel, lane 1), as
expected. Essentially all of the minigenome which accumulated
in the absence of L was also sensitive to degradation (Fig.

62   -

7A, upper panel, lane 2). Plasmid-derived minigenome
synthesized in the absence of L, and in the presence of N and
P alone, did not appear to occur efficiently.

When the complete set of three support plasmids was
5   present, significant amounts of micrococcal nuclease-resistant
minigenome RNA accumulated for each of the minigenomes (Fig.
7A, upper panel). As was the case with the positive-sense
RNAs, the greatest amount of progeny minigenome was observed
with the +2 minigenome. The +1 and +3 minigenomes were next in
10   abundance, with levels of genomic RNA that were 67% and 42% of
that of the +2 minigenome.

The foregoing examples demonstrate that the HPIV3 N,
P and L proteins were necessary and sufficient for efficient
transcription and RNA replication. The very robust nature of
15   transcription and RNA replication mediated by the
reconstituted PIV3 polymerase confirmed the functionality of
the encoded proteins. It is further expected that inclusion
of additional viral proteins within the expression system will
augment or modify these processes. Coexpression of PIV C, D
20   and potentially V, within the compositions and methods of the
invention will be useful, e.g., to augment and/or modify RNA
replication. For this purpose, plasmids will be constructed
and assayed according to the foregoing methods to achieve
coexpression of one or more of these elements to determine
25   their effects on PIV transcription and RNA replication, as
well as on PIV phenotype in suitable infection models.

EXAMPLE VI
30   Construction of Infectious, Recombinant PIV from cDNA

The following examples describe production of
infectious, recombinant PIV (rPIV) by intracellular
co-expression of four plasmid-borne cDNAs. These cDNAs
35   separately encode a complete HPIV3 genome and the HPIV3
nucleocapsid protein N, the phosphoprotein P, and the
polymerase protein L.

63

A)   Viruses and cells

Modified vaccinia strain Ankara (MVA), the vaccinia
virus recombinant that expresses bacteriophage T7 RNA
polymerase, was provided according to Wyatt et al., Virol.
210:202-205, 1995, incorporated herein by reference in its
entirety. HEp-2 monolayer cultures were maintained at 37° C
in 5% CO_2 with OptiMEM 1 (Life Technologies) supplemented with
2% FBS, 50μg/ml gentamicin sulfate and 2mM glutamine. The JS
wt strain of HPIV3 and its attenuated ts derivative, JS cp45,
were propagated in LLC-MK2 cells as described by Hall et al.,
Virus Res. 22: 173-184, (1992), incorporated herein by
reference in its entirety.

B)   cDNAs

The full cDNA clone designated p218(131) (Fig. 1,
SEQ ID NO: 1) encoding a complete genomic sequence of HPIV3
was constructed as described above. A second cDNA clone
designated p3/7(131) (SEQ ID NO: 14) (deposited under the
terms of the Budapest Treaty with the ATCC, and granted the
designation 97990) was constructed to encode a complete
antigenomic sequence of HPIV3. p3/7(131) differs from
p218(131) in that the positions of the T7 promoter and the
ribozyme/T7 terminator relative to the HPIV3 cDNA insert have
been interchanged. Thus, the first nucleotide synthesized in
p3/7(131) is the 5´ end of the antigenome, namely the
positive-sense complement of genome position 1, and the 3´
antigenome end defined by ribozyme cleavage is the complement
of genome position 15462. A third clone designated
p3/7(131)2G (SEQ ID NO: 15) (deposited under the terms of the
Budapest Treaty with the ATCC, and granted the designation
97989) was also constructed identical to p3/7(131), except
that two G residues were inserted between the 5´ end of the
antigenome and the T7 promoter.

For construction of p/37(131)2G and p3/7(131), the
two plasmids p(A*A´CC´D*E) and p(E*FF´GHIJKL*) were modified
and joined to encode the complete positive-sense antigenome of
HPIV3. First, the T7 terminator and delta ribozyme abutting
the 3´ end of HPIV3 in p(A*A´CC´D*E) were replaced by a T7
promoter using PCR (see Fig. 8). The positive-sense primer

64

5´-GGCCCGTC*GACGCG*TAATACGACTCACTATAGG<u>ACCAAACAAGAG</u>-3´ (SEQ ID
NO: 18) placed the T7 promoter (bold) adjacent to the 3´
leader of HPIV3 (HPIV3 sequence underlined) with 2 G residues
inserted between these two elements to improve transcription.

5   A unique *Mlu* I site (italicized) was placed upstream of the
T7 promoter for cloning purposes (Fig. 8). The negative-sense
primer 5´- ^{1224}CGGCAT*CACGTG*CTAC^{1209}- 3´ (SEQ ID NO: 19) spanned
nt 1209-1224 of the HPIV3 N gene and included a unique *Pml* I
site (italicized) present in the natural HPIV3 sequence. Both

10   the PCR product and the parent template p218(A*A´CC´D*E) were
digested with *Mlu* I and *Pml* I and the PCR product was then
cloned into the *Mlu* I-*Pml* I window. A second PCR reaction was
done using the same negative-sense primer and a positive-sense
primer without 2G residues inserted between the T7 promoter

15   and 3´ end of HPIV3. These plasmids were designated p(Left +
2G) and p(Left +). The construction of p(Left + 2G) is
illustrated in Fig. 8, the construction of p(Left +) followed
the same strategy.

          Plasmid p(E*FF´GHIJKL*) was modified by PCR to place
20   the delta ribozyme and T7 terminator adjacent to the 5´ end of
HPIV3 (Fig. 9). The positive-sense primer:
5´-GGATTT*GCGCGC*^{14813}<u>*AATTTAAAT*CATCTGG</u>^{14828}-3´ (SEQ ID NO: 16)
introduced a *BssH* II site (italicized) just upstream of a
unique *Swa* I site (italicized, underlined) in the L gene of

25   HPIV3 (HPIV3 specific sequence is underlined). The
negative-sense primer:
5´-CCCAGGT*CGGACC*GCGAGGAGGTGGAGATGCCATGCCAGCCC^{15462}<u>ACCA</u>
<u>AAACAAGAGAAGAACTCTGTTTGG</u>$^{15435-}$ 3´ (SEQ ID NO: 17) placed a
portion of the delta ribozyme (bold) which included a

30   naturally-occurring unique *Rsr* II site (italicized) adjacent
to the 5´ end of the HPIV3 genome (negative-sense underlined).
This PCR product was digested with *BssH* II and *Rsr* II and
cloned into the *BssH* II-*Rsr* II window of plasmid p3/7 (Fig.
9). Plasmid p3/7 is identical to p218 except that the delta

35   ribozyme and T7 terminator are in the reverse orientation.
The p3/7 plasmid containing the above-mentioned insertion was
designated pPIV3-3/7 and contained the complete delta ribozyme
and T7 terminator adjacent to 5´ terminal 651 nucleotides of

HPIV3. The *Swa*I and *NgoM*I fragment of plasmid pPIV3-3/7 was
then isolated and cloned into the *Swa*I-*NgoM* I window of
p(E*FF´GHIJKL*). The resulting plasmid, designated p(Right +)
placed the complete delta ribozyme and T7 terminator adjacent
5   to the 5´ end of HPIV3 (see Fig. 9). The *Xho* I -*NgoM* I
fragment of p(Right +) was cloned into the *Xho* I -*NgoM* I
window of p(Left +) and p(Left + 2G) resulting in plasmids
p3/7(131) (SEQ ID NO: 14) and p3/7(131 2G) (SEQ ID NO: 15),
respectively (Fig. 10). These each encode the complete
10   positive-sense analog of HPIV3 antigenomic RNA, with the
latter cDNA containing two G residues adjacent to the T7
promoter for improved transcriptional efficiency.

       C)   Transfection

       HEp-2 cells were grown to 90% confluence in six well
15   plates. Each well of a six-well plate (1.5 X 10^6 cells) was
transfected with the three previously-described support
plasmids, 0.4μg pTM(P), 0.4μg pTM(N), 0.05μg pTM(L), together
with 5μg of full-length genomic or antigenomic HPIV3 cDNA.
The plasmids were added to 0.2 ml of OptiMEM 1 (Life
20   Technologies) containing 12μl of LipofectACE (Life
Technologies). After an incubation period of approximately 15
minutes at room temperature, 0.8 ml of OptiMEM 1 containing 2%
fetal bovine serum and 1.5 X 10^7 pfu of MVA-T7 was added to
each well. The cultures were incubated at 32°C for 12 hours,
25   after which the media was replaced with fresh OptiMEM 1
containing 2% fetal bovine serum. The cultures were incubated
at 32°C for three additional days then harvested and passaged
(referred to as passage 1) onto fresh HEp-2 monolayers. These
passage 1 cultures were incubated at 32°C for five days, and
30   virus present in the culture was harvested, passaged once in
LLC-MK2 cultures, and characterized by
hemagglutination-inhibition (HAI) as described (van Wyke
Coelingh et al., <u>Virol.</u> 143: 569-582, (1985) (incorporated
herein by reference in its entirety) to determine if it
35   possessed the monoclonal antibody resistant mutation (MARM)
that marked virus recovered from cDNA.

66

D)   Sequencing of Recombinant Virus

          The presence of nucleotide sequence markers in the
HN and L genes of recombinant PIV was determined by RT-PCR of
RNA isolated from recovered virions. 1 ml of rPIV (1 X
5   10^5pfu/ml, passage level 2) was precipitated with 200μl 25%
polyethylene glycol by incubation on ice for one hour and
centrifuging at 12,000g for 15 minutes. The RNA was purified
with TRIzol reagent (Life Technologies) following the
manufacturer's recommended procedure. RT-PCR was performed
10   with the Superscript kit (Life Technologies) following the
manufacturer's recommended protocol. Control reactions were
identical except that reverse transcriptase was omitted from
the reaction to confirm that the PCR products were derived
solely from virus RNA and not from possible contaminating cDNA
15   plasmids. Four primer pairs were used to generate PCR
products from nt 7334-8715, 9364-10854, 10939-15392, and
13623-15392. The resultant PCR products were then sequenced
using cycle dideoxynucleotide sequence analysis (New England
Biolabs, Beverly, MA).
20

                              EXAMPLE VII

                 Recovery of Recombinant Virus From cDNA Encoding
                          Negative-Sense Genomic RNA

25   Plasmid p218(131) and the three support plasmids
pTM(N), pTM(P), and pTM(L) were transfected into HEp-2 cells
with MVA expressing T7 RNA polymerase. A control group
consisting of pTM(N), pTM(P), pTM(L), and MVA was
cotransfected into HEp-2 cells without p218(131). On day
30   four, the transfection was harvested, passaged onto fresh
HEp-2 cell monolayers for five days, and passaged again for 5
days in LLC-MK2 cultures (passage 2). Virus present in the
passage 2 harvest was further characterized by HAI. Cultures
from the transfection group which received the three support
35   plasmids without the full-length genomic clone p218(131) did
not yield HPIV3. The rPIV recovered virus was confirmed to be
HPIV3 since it reacted in the HAI assay with the mAbs 77/5,
101/1, and 454/11 which are specific for HPIV3 (Table 1). It

was presumptively identified as being cDNA-derived because it failed to react with mAbs 170/7 and 423/6, consistent with the MARM mutation which had been introduced into the cDNA.

TABLE 1

rJS-NS Contains the MARM Mutation Introduced into the
p218(131) Full-Length Negative Sense cDNA

Virus	Hemagglutination-inhibiting Antibody Titer (Reciprocal) of Indicated mAb				
	77/5[1]	101/1[1]	454/11[1]	170/1[2]	423/6[2]
JS wt[3]	800	6400	6400	25,600	25,600
rJS-NS[4]	3200	25,600	6400	≤ 25	≤ 25

[1] mAb 77/5 recognizes antibody epitope IIB, mAbs 101/1 and 454/11 recognize antibody epitope IIIA of HN glycoprotein, all of which were not altered in p218(131).

[2] Mabs 170/7 and 423/6 which both recognize antibody epitope I of JSwt, fail to recognize rJS due to the MARM mutation at this site.

[3] Biologically derived wild type HPIV3 JS.

[4] Recombinant JS virus derived from negative-sense genomic cDNA.

To confirm that rPIV was indeed recovered from cDNA, it was analyzed in parallel with wild-type JS strain HPIV3 by RT-PCR using four primer pairs flanking the seven inserted marker mutations. Each PCR product obtained was dependent

5   upon the inclusion of RT, indicating that each was derived from RNA and not from contaminating cDNA. Cycle-sequencing of the four PCR products confirmed that the sequence of the rPIV contained each of the seven markers, sequencing data showing three of the markers is illustrated in Figure 11. The

10   sequence differences between rPIV and JS wt in the HN gene, including nt 7903, 7913, and 7915 are readily apparent. Similar sequence analyses were carried out for the other four markers at nt positions 7593, 10355, 11333, and 15248, and the rPIV possessed each mutation.

15   These results demonstrate successful recovery of infectious rPIV from cDNA encoding a negative-sense genomic RNA. This differs from most published reports for recovery of nonsegmented negative strand RNA viruses, in which the cDNA used for virus recovery had been designed to encode

20   positive-sense antigenomic RNA (Baron and Barrett, supra, 1997; Collins et al., supra, 1995; Conzelmann, supra, 1996; Garcin et al., supra, 1995; Lawson et al., supra, 1995; Radecke et al., supra, 1995; Whelan et al., supra, 1995). The recovery of infectious virus from a cDNA encoding genomic RNA

25   had previously been reported only in the case of Sendai virus, and the efficiency of recovery was much lower than for cDNA encoding antigenomic RNA (Kato et al., 1996). In most other studies, the recovery of virus was achieved with antigenomic cDNA but not with genomic cDNA (Lawson et al., 1995; Whelan

30   et al., 1995). A number of potential problems have been noted which may explain these refractory results, including possible annealment of cDNA-encoded genomic RNA with mRNA produced by the support plasmids; resulting in inactive hybrids (Conzelmann, supra, 1996; Lawson et al., supra, 1995).

35   It has also been noted that T7 RNA polymerase appears to terminate preferentially at the gene junctions of genomic RNA, perhaps because the oligo U tract of the GE signal resembles

the natural signal for transcription termination by T7 RNA
polymerase (Whelan et al., _supra_, 1995).

5   **EXAMPLE VII**

                 <u>Recovery of Recombinant Virus From cDNA Encoding</u>
                        <u>Positive-Sense Antigenomic RNA</u>

                 As described in more detail above, p3/7(131) and
10   p3/7(131)2G were constructed to encode a positive-sense,
        antigenome that give rise to recombinant PIV. Plasmid
        p3/7(131)2G is identical to p3/7(131) but for the addition of
        two G residues between the T7 promoter and the first
        nucleotide of the antigenome. The addition of two G residues
15   between the T7 promoter and the first HPIV3 nucleotide
        p3/7(131)2G is based on the preceding examples demonstrating
        that the presence of the two added G residues (as opposed to
        0, 1 or 3 added G residues) yielded substantially increased
        levels of minireplicon replication.
20   The two antigenome cDNAs [p3/7(131) and p3/7(131)2G]
        were transfected separately into cells together with the N, P
        and L support plasmids, and were infected simultaneously with
        the MVA-T7 recombinant virus using the same procedure
        described above for p218(131). Infectious virus from each
25   antigenomic cDNA was recovered and was presumptively
        identified as being cDNA-derived by its inability to react
        with mAbs 423/6 and 170/7.
                 The efficiency of virus recovery was evaluated for
        each of the antigenome cDNAs versus the genome cDNA p218(131).
30   Twelve transfection reactions using the negative-sense genome
        cDNA p218(131) (SEQ ID NO: 1) were conducted in parallel with
        twelve transfections using the positive-sense antigenome cDNA
        p3/7(131)2G (SEQ ID NO: 15) to compare efficiency of virus
        recovery from the two cDNAs. One ml of the transfection
35   harvest from each well was titered on LLC-MK2 cells and the
        remaining 2mls were passaged (passage 1) onto fresh LLC-MK2
        cells. At day five, passage 1 was harvested and titered as
        described above. Recombinant virus was recovered from 12/12

wells transfected with p3/7(131)2G but from only 4/12 wells
transfected with p218(131). The mean titer of virus present
in culture of the passaged virus from the positive-sense
antigenome was $10^{5.0}$, nearly ten-fold higher than that from the
negative-sense genome, which was $10^{4.1}$. However, with one
additional amplification the titers became equivalent.

TABLE 2

Comparison of the Efficiency of Recovery of Recombinant Virus from cDNAs Encoding Genomic or Antigenomic RNA, the latter with or without 2G residues adds to the T7 promoter.

Experiment#	Transfected cDNA	Sense of Encoded RNA	Rescue Efficiency[1]	Mean Titer ($\log_{10}$ pfu/ml) of Recovered Virus[2]
#1	p3/7(131)2G[3]	Antigenomic	12/12 (100%)	5.0 ± 0.27
	p218(131)	Genomic	4/12 (33%)	4.1 ± 0.27
#2	p3/7(131)2G[3]	Antigenomic	6/6 (100%)	6.6 ± 0.18
	p3/7(131)	Antigenomic	12/12 (100%)	5.3 ± 0.12
	p218(131)	Genomic	5/5 (100%)	4.9 ± 0.19

1   Number of transfection cultures yielding rJS/number tested.

2   The mean Titer ± standard error was determined following one passage of transfection harvest for five days in LLC-MK2 cells.

3.   Contains 2 G residues between the T7 promoter and the 5' end of the antigenome.

73   −

The efficiency of recovery of recombinant virus from
the three full-length plasmids encoding the genomic or
antigenomic HPIV3 RNAs was next studied, (i) to determine
whether genomic or antigenomic cDNA is more efficient at
5   generating recombinant virus and (ii) to determine the effect
of two extra 5' terminal G residues on the yield of
recombinant viruses (Table 2.) Unfortunately, it was not
possible to directly titer the transfection harvest by plaque
titration because residual MVA-T7 interfered with plaque
10   formation of rJS on LLC-MK2 monolayer cultures. Therefore, we
first compared the efficiency of recovery of rJS from multiple
independent transfections and, as seen in other experiments,
found that rJS was more frequently recovered from cultures
transfected with p3/7(131)2G than from cultures transfected
15   with p218(131). Since each transfection harvest, including
those with p218(131) yielded rJS in other experiments, it was
not possible to use this method of analysis to compare the
relative efficiency of generating recombinant virus. We
therefore estimated the relative quantity of virus present in
20   the transfection harvest by quantitating the amount of virus
following one passage of the transfection harvest (Table 2).
Since each plasmid was designed to yield an identical virus
(rJS), differences in titers of the passaged virus were
considered a reflection of differences in the titer of virus
25   present in transfection harvest. In the above experiments,
the construct with two 5' terminal G residues was the most
efficient in generating rJS. This indicated that the two
added residues indeed increased efficiency of recovery, but
the mechanism for this small increment in efficiency was not
30   defined. These findings indicate a small, but measurable,
advantage of using p3/7(131)2G as substrate for future
experiments designed to introduce mutations into the HPIV3
cDNA. The small advantage in recovery of recombinant virus
from p3/7(131)2G might be especially needed to recover viruses
35   that have attenuating mutations that restrict replication in
vitro. Although VSV has been recovered from antigenomic cDNA
which included three G residues at the 5'-terminal end, the
efficiency of recovery of virus from this construct was not

74

compared with recovery from the same construct lacking the
extra residues (Whelan et al., Proc. Natl. Acad. Sci. USA
92:8388-8392 (1995), incorporated herein by reference). In
contrast to the experience with Sendai virus and measles

5   virus, it is clear that extra-viral G residues at the 5'-
terminal end of the antigenome are not deleterious to recovery
of recombinant virus, and, in fact, appear to be advantageous
[Garcin et al., supra, 1995; Radecke et al., supra, 1995; Kato
et al., 1996). The genomic and antigenomic plasmids without

10   two 5'-terminal guanine residues appeared equally efficient in
generating recombinant virus. This is in contrast to previous
reports which have found genomic cDNA to be less efficient
than antigenomic cDNA in generating recombinant virus (Whelan,
1995; Kato et al., 1996).

15   The efficiency of recovery of virus from p3/7(131)
(SEQ ID NO: 14) was compared to that of p3/7(131)2G (SEQ ID
NO: 15 and p218(131) (SEQ ID NO: 1) in a similar experiment as
above. Recovery from p3/7(131) was comparable to that of
p217(131). Recovery was more consistent and efficient with

20   both antigenome cDNAs containing the extra Gs (Table 2).

EXAMPLE VII
Efficiency of plaque formation of rPIV at permissive and
restrictive temperatures

25

           The level of temperature sensitivity of replication
in vitro of control viruses and rPIV derived from both
p218(131) and p3/7(131)2G was determined at 32°C, 37°C, 39°C,
and 40°C in LLC-MK2 monolayer cultures as previously described

30   (Hall et al., Virus Res. 22: 173-184, (1992), incorporated
herein by reference in its entirety), with the following
modifications. Viruses were inoculated onto LLC-MK2
monolayers on 24-well plates in serial 10-fold dilutions
allowed to adsorb for one hour at room temperature. The

35   cultures were then overlaid with 1ml of L-15 supplemented with
2mM glutamine and 0.8% methylcellulose and the plates were
then incubated for 5 days at the indicated temperature. The
methylcellulose overlay was removed and the monolayer fixed

with 80% methanol at 4°C for 1h. The viral plaques present in
the monolayer were scored using a mixture of two
HPIV3-specific anti-HN murine mAbs 101/1 and 66/4 as ascites
fluid used at a dilution of 1:500, using an immunoperoxidase
method of staining specific for murine antibodies as
previously described (Murphy et al., Vaccine 8: 497-502
(1990), incorporated herein by reference in its entirety).

Recombinant virus derived from either positive or
negative-sense cDNA were characterized by plaque assay at
32°C, 37°C, 39°C, and 40°C to determine if they were
phenotypically similar to JS wt virus. Both positive and
negative-sense rPIV were comparable to the JS wt virus in
their level of replication at elevated temperatures of 39°C
and 40°C (Table 3). This is in contrast to the ts mutant JS
cp45 which exhibits a 30-fold reduction in titer at 37°C and
fails to produce plaques at 39°C or 40°C.

TABLE 3

The rJS Resembles its Biologically Derived Parent
JS Wild-Type Virus in the Level of Replication at
Restrictive Temperature (39°C - 40°C)

Virus	Virus Titer (log 10 pfu/ml)			
	32°C	37°C	39°C	40°C
rJS-PS[1]	6.1	6.1	6.1	6.6
rJS-NS[2]	6.9	7.1	7.1	7.0
JScp45[3]	6.3	4.3	<0.7	<0.7
JS wt	6.5	6.8	6.6	6.7

[1]   Recombinant virus derived from the antigenomic-sense
       clone p3/7(131)2G

[2]   Recombinant virus derived from the genomic-sense clone
       p218(131)

[3]   JScp45 is a temperature sensitive mutant derived from JS
       wt.

The sequence of JS cp45 has been fully determined (Stokes et al., supra, 1993) and mutations have been identified in the leader, N, P, M, F, HN, and L genes. However, it is unknown which mutation(s) are responsible for

5   the ca, att, or ts phenotypes. Because exemplary rPIV of the invention demonstrate the ts⁺ phenotype like the JS wt parent, cp45 mutations among other mutations known or yet to be discovered for PIV can be introduced, alone or in combination, into the full-length cDNA to pinpoint the effects of

10 individual mutations or combinations thereof, e.g., by evaluating replication of the recombinant virus incorporating the mutation(s) of interest at elevated temperatures. The mutation(s) thus identified can be incorporated into effective, attenuated vaccine agents as described in the

15 Examples below. These and other mutations incorporated into recombinant PIV can be further optimized by, e.g., mutagenesis to create two or more nucleotide substitutions per codon to render a recombinant virus that is more genetically stable than a biologically derived mutant strain.

20

EXAMPLE VIII
Replication of rPIV in Hamsters

25   Thirty-six 16-week-old golden Syrian hamsters were divided into four groups of nine and inoculated intranasally with 0.1ml containing $10^{5.5}$ pfu of either rPIV recovered from negative-sense cDNA, rPIV recovered from positive-sense cDNA, JS cp45, or JS wt virus. On day 4, the hamsters were

30 sacrificed and the lungs and nasal turbinates harvested. The lungs were homogenized in a 20% w/v L-15 suspension containing 2.5μg/ml amphotericin B (Quality Biologicals, Gaithersburg, MD), 200μg/ml pipericillin (Lederle Laboratories, Pearl River, NY), and 50μg/ml gentamicin (Quality Biologicals). The nasal

35 turbinates similarly were homogenized in a 10% w/v L-15 suspension. After homogenization, the samples were aliquoted and rapidly frozen in a dry ice-ethanol bath. Virus present in the samples were titered at a later date in 96 well plates

77

of LLC-MK2 cells at 32°C scoring CPE at five and seven days after inoculation. The mean $\log_{10}$ $TCID_{50}$/gm was calculated for each group of nine hamsters.

Table 4 illustrates that rPIV recovered from negative-sense cDNA, rPIV recovered from positive-sense cDNA replicate to substantially the same level as the JS wt in the upper and lower respiratory tract of hamsters. This is in contrast to the JS cp45 virus, which is attenuated at each site.

TABLE 4

The rJS Resembles its Biologically Derived Parent
JS wt Virus in the Level of Replication in the
Upper and Lower Respiratory Tract of Hamsters.

Virus	Mean Virus Titer ($\log_{10}$ $TCID_{50}$/g)[5]	
	Nasal Turbinates	Lungs
rJS-PS[1]	6.6 ± 0.2	4.1 ± 0.3
rJS-NS[2]	6.4 ± 0.1	4.2 ± 0.2
JScp45[3]	4.2 ± 0.2	≤ 1.4 ± 0.0
JS wt[4]	6.3 ± 0.2	4.6 ± 0.3

[1]   Recombinant virus recovered using p3/7(131)2G encoding the positive-sense HPIV3 antigenome.

[2]   Recombinant virus recovered using p218(131) encoding the negative-sense HPIV3 genome.

[3]   Biologically derived ts mutant.

[4]   Biologically derived parent virus.

[5]   Mean titers ± standard errors for nine hamsters per group.

Thus, exemplary rPIVs of the invention can retain the replicative capacity in hamsters exhibited by the biologically derived JS wt parent strain, whereby mutations such as those present in the JS cp45 candidate vaccine that restrict replication in hamsters and other hosts, including

78

non-human primates and humans, can be identified and
incorporated within modified rPIV strains of the invention, as
described in the Examples below.

5

EXAMPLE IX

Identification of Amino Acid Substitutions in HPIV3 Specifying Attenuated Phenotypes, and Incorporation of Attenuating Mutations into Infections, Attenuated PIV Clones

10   The ability to generate infectious PIV from cDNA
facilitates development of live-attenuated parainfluenza virus
vaccines. More specifically, by using the methods and tools
disclosed herein the genetic basis of attenuation of PIV
candidate vaccines can be readily determined, and infectious
15   PIV vaccines produced from cDNA can be designed to achieve a
finely calibrated level of attenuation and immunogenicity.
          In addition, the tools and methods of the invention
provide for vaccine development for all three human
parainfluenza viruses, HPIV1, HPIV2 and HPIV3 that are most
20   important in human disease. For example, to produce and
select effective HPIV3 vaccine agents within the invention,
mutations associated with desired phenotypes of biologically
deriving HPIV3 candidate vaccines or the attenuated BPIV3
virus, e.g. attenuating mutations, can be identified and
25   incorporated into rPIV. Applying these methods, attenuating
mutations from a large menu of candidate mutations are
selected and combined to generate rPIV having a desired
balance between attenuation and immunogenicity, and which
retain the attenuation phenotype following replication in
30   humans.
          In the present example, the genetic bases of
temperature-sensitive (ts) and in vivo attenuation (att)
phenotypes of the PIV3 JS cp45 live-attenuated virus are
described. Seven exemplary recombinant PIV3 viruses (three
35   single-, three double-, and one triple-lesioned virus) were
recovered from full-length antigenomic cDNA and analyzed for
their ts and att phenotypes. These recombinants bore one or
more amino acid substitution mutations present in the L gene

of JS *cp*45 (alternatively referred to herein as *cp*45), adopted
within a cDNA clone of the JS wt parent. These three
exemplary, biologically derived mutations are all present in a
representative strain of JS *cp*45 grown in Vero cells,
designated JS *cp*45 Vero, deposited on August 21, 1997 under
the terms of the Budapest Treaty with the American Type
Culture Collection (ATCC) of 12301 Parklawn Drive, Rockville,
Maryland 20852, U.S.A., and granted the accession number ATCC
VR 2588.

Analyses of exemplary PIV recombinants, presented
below, demonstrate that each of the three exemplary mutations
in L (Tyr$_{942}$ to His, Leu$_{992}$ to Phe, and Thr$_{1558}$ to Ile) contribute
to the *ts* and *att* phenotypes of *cp*45 and are useful for
generating of recombinant vaccine virus.

Viruses and cells.

The PIV3 JS wt and *cp*45 viruses were grown in
LLC-MK2 cells as described previously (Hall et al., <u>Virus Res.</u>
22:173-184 (1992), incorporated herein by reference). The
vTF7-3 recombinant vaccinia virus is described in Fuerst et
al., <u>Virology</u> 225: 419-422 (1996) and the modified vaccinia
virus Ankara (MVA) which expresses the T7 polymerase is
described in Wyatt et al., <u>Virology</u> 210:202-205 (1995) (each
incorporated herein by reference). HEp-2 (ATCC CCL 23) and
LLC-MK2 (ATCC CCL 7.1) cells were maintained in OptiMEM (Life
Technologies) supplemented with 2% FBS and gentamicin sulfate
(50ug/mL).

**Construction of point mutations in the L gene of
PIV3.**

pUC19 was modified to accept a fragment of the JS wt
PIV3 L gene in order to introduce point mutations into the L
gene by site-directed mutagenesis. First, a unique *Nhe* I
restriction site was introduced into pUC19 by ligating a pair
of complementary oligonucleotides (5' GATCGATGCTAGCCC 3' (SEQ
ID NO: 23) and 5' GATCGGGCTAGCATC 3' (SEQ ID NO: 24))
containing an *Nhe* I restriction site into the *Hind* III site of
pUC19 to create pUC19 (N). The *Sph* I (PIV3 nt 11317) to *Nhe* I
(PIV3 nt 14087) fragment of pTM(L), which includes the

positions where the three coding changes in *cp45* occur and
which can be directly introduced into the full-length PIV3
cDNA (see below), was cloned into the *Sph* I and *Nhe* I site of
pUC19 (N) to create pUCL(N-S). Point mutations were

5   introduced into pUCL(N-S) using mutagenic oligonucleotides
with the Transformer mutagenesis kit (Clontech, Palo Alto, CA)
for the purpose of (i) creating exemplary amino acid
substitutions at L protein positions 942, 992, and 1558,
individually and in combination, and (ii) ablating one

10   specific naturally-occurring restriction enzyme site proximal
to each codon substitution as a marker [See Table 5].

TABLE 5: Nucleotide substitutions introduced into rPIV3 that encode cp45L protein gene amino acid substitutions and, as markers, ablate naturally-occurring restriction enzyme sites.

rPIV3 designation	Amino Acid Substitution (wt to cp45)	Sequence of wt[a]	Sequence of Mutant	Restriction Enzyme site Ablated
r942	Tyr-942 to His	11468-TTACATGGCCAT (SEQ. ID NO: 25)	11468-TCACATGGCCGAT (SEQ. ID NO: 26)	Eae I
r992	Leu-992 to Phe	11618-TTTTGATTGGGC (SEQ. ID NO: 27)	11618-TTTTGATTGGGC (SEQ. ID NO: 28)	Bsr I
r1558	Thr-1558 to Ile	13307-TGGTCCTAATACTG (SEQ. ID NO: 29)	13307-TGGGCCTAATATCG (SEQ. ID NO: 30)	Ava II

a. The nucleotide sequence around each of the three mutated regions is shown. The first nucleotide in each provided sequence is numbered according to its position in the complete antigenomic RNA. The codon involved in each amino acid substitution is in bold. Naturally-occurring restriction enzyme sites present in the wt sequence, and which were ablated to mark the mutation, are in italics. The nucleotides that were mutated to produce an aa substitution or remove a restriction enzyme site are underlined.

Mutations introduced in pUCL(N-S) derivatives were verified by
dideoxynucleotide sequencing of plasmid DNA. The *Sph* I to
*Bam*HI (nt 13733) fragment of pUCL(N-S) containing the *cp*45
individual L gene mutations was subcloned into the *Sph* I to

5   *Bam*HI sites of pTM(L) to give pTM(L)-942, -992, -942/992, and
-1558; the other double and triple mutations were assembled
using the *Pin AI* and *Nhe I* sites (Fig. 12). The mutant pTM(L)
plasmids were each tested at permissive temperature (32°C) for
the ability to direct the expression of the chloramphenicol

10   acetyl transferase marker gene in a minireplicon system
comprising a plasmid-encoded minigenome RNA and the N, P and L
proteins (Durbin et al., <u>Virology</u> 234:74-78 (1997),
incorporated herein by reference). The various mutant L
plasmids supported marker gene expression to 75-106% the level

15   of wt L, indicating that each engineered cDNA was free of
significant spurious mutation (not shown). The *Sph* I to *Nhe* I
fragments of each of the mutant pTM(L) plasmids were then
subcloned into the *Sph* I to *Nhe* I window of the full-length
PIV3 JS antigenomic cDNA p3/7(131)2G to create seven

20   full-length PIV3 cDNA clones representing every possible
combination of the three substitutions.

**Recovery of recombinant PIV3 (rPIV3) bearing one,
two or three *cp*45 L protein substitutions.**

Each full-length antigenomic cDNA bearing one or

25   more *cp*45 L gene mutations, together with the three support
plasmids pTM(N), pTM(P) and pTM(L), was transfected into HEp-2
cells on 6-well plates (Costar, Cambridge, MA) using
LipofectACE (Life Technologies) and MVA-T7 as described above.
After incubation at 32°C for 4 days, the transfection harvest

30   was passaged onto HEp-2 cells on 6-well plates which were
incubated at 32°C for 4 days. Each passage 1 supernatant was
harvested and passed onto a T-25 flask of LLC-MK2 cells, which
was incubated at 32°C for 5-6 days. The passage 2 supernatant
was harvested and the presence of recombinant virus was

35   initially confirmed by immunoperoxidase staining of virus
plaques (Murphy et al., <u>Vaccine</u> 8:497-502 (1990), incorporated
herein by reference) with anti-HN monoclonal antibody (Mab)
77/5, which binds to both biologically derived and recombinant

JS PIV3, and Mab 423/6, which does not bind to cDNA-derived
virus because its epitope was ablated to serve as a marker.
Virus present in passage 1 was subjected to two or three
rounds of plaque purification on LLC-MK2 cells as described
5   previously. Each biologically cloned recombinant virus was
amplified twice in LLC-MK2 cells at 32°C to produce virus for
further characterization. Virus was concentrated from
clarified medium by polyethylene glycol precipitation, and
viral RNA (vRNA) was extracted with Trizol Reagent (Life
10   Technologies). Reverse transcription (RT) was performed on
vRNA using the Superscript II kit with random hexamer primers
(Life Technologies). The Advantage cDNA PCR kit (Clontech,
Palo Alto, CA) and sense (5' nt
11190-GCATTATCTAGATGTGTCTTCTGGTCAGAG 3' nt-11219) (SEQ ID NO:
15   31) and antisense (5' nt 14140-CCTGAATTATAATAATTAACTGCAGGTCCT
3' nt-14111) (SEQ ID NO: 32) primers specific for the PIV3 L
gene were used to amplify the region spanning the *Sph* I to *Nhe*
I fragment. The PCR fragments were analyzed by digestion with
each of the restriction enzymes whose recognition sites had
20   been ablated during insertion of the three *cp*45 mutations in L
(see Table 5).

**Efficiency of plaque formation (EOP) at permissive
and restrictive temperatures of rPIV3 bearing one, two or
three *cp*45 L protein amino acid substitutions.**

25   The level of temperature sensitivity of plaque
formation *in vitro* of control and recombinant viruses was
determined at 32°C, 37°C, 38°C, 39°C, 40°C, and 41°C in LLC-MK2
monolayer cultures and plaques were enumerated by
hemadsorption with guinea pig red blood cells following
30   removal of the methylcellulose overlay. Alternatively, viral
plaques present in the monolayer were identified by
immunoperoxidase staining with a mixture of two PIV3-specific
anti-HN murine mAbs 101/1 and 454/11 diluted 1:500, (Murphy et
al., _supra_, (1990)).

35   **Hamster studies.**

                4 to 16 week-old golden Syrian hamsters in groups of
six were inoculated intranasally with 0.1ml OptiMEM1 per
animal containing $10^{5.5}$ pfu of rPIV3 JS wt, PIV3 *cp*45 virus, or

one of the rPIV3 containing one or more *cp*45 L protein substitution(s). On day 4 post-infection, the hamsters were sacrificed, the lungs and nasal turbinates were harvested, and the virus was quantified as described above. The mean $\log_{10}$ $TCID_{50}/g$ was calculated for each group of six hamsters.

RESULTS

Introduction of the PIV3 *cp*45 L protein amino acid substitutions into wt JS rPIV3.

As noted above, the three amino acid substitutions present in the L protein of *cp*45 (Table 5) were introduced individually or in selected combinations into the antigenomic cDNA that encodes its wt parent, PIV3 JS strain. Each introduced mutation was engineered to be marked with a silent mutation that ablated a proximal, naturally-occurring restriction enzyme site to facilitate monitoring the mutation in recovered rPIV3 (Table 5, Fig. 12). The coding change at amino acid 1558 was designed to contain two nucleotide changes in r1558, compared to the one nt substitution in *cp*45, to reduce the chance of reversion at this site during *in vitro* or *in vivo* replication.

Seven rPIV3s bearing one, two or all three of the amino acid substitutions from *cp*45 were recovered in tissue culture by transfection of each antigenomic cDNA together with the pTM(N), pTM(P) and pTM(L) support plasmids and coinfection with the vaccinia virus MVA/T7 pol recombinant (Wyatt et al., supra, (1995)). Each rPIV3 possessed the Mab resistance marker that had been deliberately introduced into the HN gene by engineering the antigenomic cDNA. The rPIV3s were biologically cloned by two or three cycles of plaque to plaque passage to ensure that each virus preparation was genetically homogeneous. This precaution was taken because vaccinia virus can meditate recombination between the antigenomic cDNA and the support plasmids.

To confirm that each of the seven rPIV3 contained the engineered mutation(s) in the L gene, RNA was purified from precipitated virions and was copied into cDNA and amplified by RT-PCR. Control reactions showed that the RT step was required for generation of RT-PCR products,

indicating that an RNA template rather than contaminating cDNA
was required for the generation of the RT-PCR product. The
RT-PCR products were subjected to digestion with the three
restriction enzymes whose recognition sequences had been

5 ablated as markers for the inserted coding changes. As
expected, the RT-PCR product of JS wt rPIV3 was cleaved the
appropriate number of times by each of the three enzymes,
whereas r942/992/1558 lacked each of the three sites ablated
during creation of the individual cp45 coding changes. Each

10 of the other rPIV3s lacked the appropriate restriction
site(s), indicating the presence of the introduced mutations.

**Efficiency of plaque formation at 32°C, 37°C, 38°C,
39°C, 40°C, and 41°C of rPIV3 bearing cp45 L mutations.**

The seven rPIV3s bearing the various combinations

15 of cp45 L protein amino acid substitutions were assayed for
their ability to form plaques on LLC-MK2 monolayers at 32°C,
37°C, 38°C, 39°C, 40°C and 41°C. As shown in Table 6, each
rPIV3 bearing a cp45 aa substitution was ts, whereas the JS wt
rPIV3 parent was not restricted in plaque formation at any

20 temperature tested. The shut-off temperature of plaque
formation of r942, r992 and r1558 was 40°C. r942 manifested a
700-fold reduction of plaque formation at 40°C, indicating
that its replication was marginally reduced at this
restrictive temperature. However, the plaque size of r942

25 also was greatly reduced at 40°C, which also indicates that
its replication was restricted at this temperature compared to
the JS wt. r942 was completely restricted in replication at
41°C (data not shown). r992 and r1558 were greatly reduced
(over a 1,000,000-fold reduction) in plaque formation at 40°C.

30 These results indicate that each of the three cp45 L gene
mutations individually specifies the ts phenotype, although
that of the r942 mutation is somewhat less restrictive. The
double mutant virus r942/1558 and the triple mutant
r942/992/1558 had a shut-off temperature of 39°C, while that

35 of r942/992 and cp45 was 38°C. The double mutant, r992/1558,
was less ts than the r992 single mutant. r992/1558, like
r942, was completely restricted in plaque formation at 41°C.
The level of temperature sensitivity exhibited by double or

triple results from a rPIVs delicate interplay of the three
mutations that cannot be predicted from level of temperature-
sensitivity exhibited by the single mutants. Also, since
r942/992/1558 was slightly less *ts* than *cp*45, other mutations
outside of the L gene also likely contribute to the *ts*
phenotype of *cp*45, therefore representing additional mutations
of interest within the invention.

Table 6: The efficiency of plaque formation (EOP) at 32°C,
37°C, 38°C, 39°C and 40°C of rPIV3 bearing one, two or three
*cp*45 L protein amino acid substitutions.

Virus[a]	pfu/ml)	Virus titer (log$_{10}$			
		32°C	37°C	38°C	39°C	40°C
r942	6.8	6.8[b]	6.6[b]	6.5[b]	4.3[b]
r992	6.9	6.8[b]	6.7[b]	6.1[b]	<0.7
r1558	6.6[b]	6.6[b]	6.4[b]	5.0[b]	<0.7
r942/992	6.7	6.5[b]	4.5[b]	3.0[b]	<0.7
r942/1558	5.2	5.0[b]	4.0[b]	1.0[b]	<0.7
r992/1558	6.7	6.7[b]	6.5[b]	5.9[a]	5.1[b]
r942/992/1558	6.6	6.2[b]	6.2[b]	2.7[b]	<0.7
cp45[b]	6.6	4.8	4.5[b]	<0.7	<0.7
rPIV3 JS	8.3	8.4	8.5	8.5	8.3

a. The *cp*45 virus is a biologically derived virus, and each
   of the other viruses tested is a recombinant.
b. Plaques were of pinpoint size.
c. Underlined numbers represent the shut-off temperature of
   plaque formation, which is defined as the lowest restrictive
   temperature at which a 100-fold reduction in titer is
   observed compared to the titer at 32°C.

Growth in hamsters. Groups of six Golden Syrian
hamsters were inoculated intranasally with JS wt rPIV3,
biologically-derived *cp*45, or with rPIV3 containing one or
more *cp*45 L protein amino acid substitutions, and virus
replication in the lungs and nasal turbinates was determined.
In this experiment [Table 7], each of the rPIV3s bearing a
single amino acid substitution was restricted in replication
in the upper and lower respiratory tract [Table 7]. However,
r942, the least *ts* virus, was only marginally suppressed in

replication in the upper and lower respiratory tract in a
second experiment. These data demonstrate that two of the
three amino acid substitutions contribute to the *att* phenotype
when present as single-lesioned recombinant viruses. However,
5   the 942 mutation indeed contributes to attenuation (e.g., the
r942/992 is more attenuated than r992 alone). Thus, each of
the amino acid substitutions in L contribute to the *att*
phenotype either acting alone or in concert with another L
amino acid mutation. Each of the double mutants was
10   attenuated indicating that loss of any of the three L gene
substitutions following replication in vivo still leaves an
attenuated virus. This is a partial explanation for the
previously observed high level of stability of the *ts*
phenotype of the *cp*45 following replication in vivo. The
15   triple mutant r942/992/1558 was as restricted as *cp*45 for
replication in the upper and lower respiratory tract
indicating that the three amino acid substitutions in the L
protein are the major contributors to the *att* phenotype of
*cp*45.

Table 7: The level of replication in the upper and lower
respiratory tract of hamsters of rPIV3 bearing one, two or
three cp45 L protein amino acid substitutions, compared to JS
wt rPIV3 and cp45[a].

Virus	Mean virus titer ($\log_{10}$ TCID$_{50}$/g ± S.E[b])	
	Nasal turbinates	Lungs
rPIV3 wt	7.4 ± .16	5.1 ± .49
r942	6.6 ± .17	3.0 ± .78
r992	4.4 ± .16	3.1 ± .11
r1558	3.8 ± .40	4.3 ± .34
r942/992	<1.5 ± 0	<1.5 ± 0
r942/1558	2.9 ± .23	1.8 ± .17
r992/1558	5.7 ± .16	3.2 ± .57
r942/992/1558	3.9 ± .15	<1.5 ± 0
cp45	4.1 ± .27	1.6 ± .08

a. Groups of six hamsters each were intranasally
    administered $10^{5.5}$ pfu of virus per animal in
    an 0.1ml inoculum, and lungs and nasal
    turbinates were harvested four days later.
b. Standard Error
c. cp45 is a biologically derived virus and the
    others are recombinant.

To summarize the above results, substitutions at L
protein amino acid positions 992 and 1558 each specified a
1,000,000-fold reduction in plaque formation in cell culture
at 40°C, while the substitution at position 942 specified a
700-fold reduction. Thus, each of the three mutations
individually contributes to the ts phenotype. The triple
recombinant which possesses all three L mutations is slightly
less ts than cp45, suggesting that there are mutations outside
of the L gene in cp45 that also might contribute to its ts
phenotype. Two of the three individual mutations in L each
contributed to restricted replication in the upper or lower
respiratory tract of hamsters, which accounts for the observed
stability of ts and att phenotypes of cp45 during replication
in vivo. Importantly, the level of temperature sensitivity of
recombinant vaccine strains in vitro was closely predictive of
attenuation in vivo. The recombinant virus possessing all
three mutations was as restricted in replication as the cp45
mutant in both the upper and lower respiratory tract of

hamsters, indicating that the L gene of the cp45 virus is a
major attenuating component of this candidate vaccine strain.
While each mutation on its own specifies the ts phenotype,
when placed together they are not simply additive but instead
5   somehow influence each other. The effect of the three
mutations together in the triple mutant seemed to ameliorate
rather than enhance the level of temperature-sensitivity
observed in the two double mutants which were evaluated.
Interestingly, this should provide an unanticipated selective
10   pressure to maintain at least some of the cp45 L mutations,
since the loss by reversion of either the 992 or 1558
substitution would increase rather than decrease the level of
temperature sensitivity. Considered together, these findings
indicate that the high level of the stability of the ts and
15   att phenotypes of cp45 virus results from the contribution of
multiple ts mutations in L to the att phenotype. The
identification of these three mutations as the major
attenuating mutations of cp45 provides the basis for
monitoring virus during all stages of manufacture and
20   following replication in humans.

It is of further interest that the tyrosine to
histidine mutation at position 942, arguably the most
conservative substitution of the three mutations, was the
least temperature sensitive. The L polymerase of PIV3 is a
25   large polypeptide, 2233 aa in length, and is thought to be a
multifunctional protein that encodes multiple domains
including those required for complex formation with the P
protein, RNA binding, RNA polyadenylation, RNA transcription
and RNA replication (Collins et al., supra, (1996)). The
30   amino acid substitutions in L at positions 942 and 992 are
located near regions that are well-conserved among other
members of the Paramyxovirus family (Blumberg et al., Virology
164:487-497 (1982); Galinski et al., Virology 165:499-510
(1988)). The mutation at position 1558 is in a region of the
35   polymerase which appears to share less sequence identity with
other L polymerases. Although the mechanism by which the ts
phenotype is conferred by the triple amino acid substitution
in L is not known, it is likely that multiple L protein

90

domains and activities are affected, or that a common
mechanism involving various activities of L is affected.

EXAMPLE X

5 ### Direct Identification, and Reconstitution into Recombinant
Vaccine Viruses, of Mutations in a Biologically Derived,
Live-Attenuated HPIV Type 3 Virus (cp45) Which Specify the
Temperature-Sensitive, Cold-Adaptation and Attenuation
Phenotypes

10   The above Examples demonstrate that each of the
three amino acid substitutions in the L polymerase protein of
*cp*45 confer the temperature-sensitive (*ts*) and attenuation
(*att*) phenotypes, but not the cold-adaptation (*ca*) phenotype
(see also, Skiadopoulos et al., J. Virol 72(3):1762-8, 1998).
15 *cp*45 contains twelve additional mutations in other proteins
(N, C, M, F and HN) or potential cis-acting sequences (the
leader region and the transcription gene start {GS} signal of
the N gene), and their contribution to these phenotypes has
been heretofore undefined. The present Example further
20 characterizes the genetic basis for the *ts, ca*, and *att*
phenotypes of *cp*45 to provide yet additional information
regarding basis for the observed high level of stability of
these phenotypes following replication of *cp*45 in humans or
non-human primates. In one aspect of this study, a
25 recombinant *cp*45 (*rcp*45) virus containing all fifteen
*cp*45-specific mutations was constructed, using a reverse
genetics system, and was found to be essentially
indistinguishable from the biologically-derived virus on the
basis of plaque size, level of temperature-sensitivity,
30 cold-adaptation, and level of replication in the upper and
lower respiratory tract of hamsters. In addition, recombinant
viruses containing: (1) the *cp*45-specific changes in the C, M,
F or HN proteins, (2) the combined leader and N gene
mutations, or (3) several combinations of the *cp*45 mutations
35 were constructed. Analysis of these recombinant viruses
showed that multiple *cp*45 mutations distributed throughout the
genome contribute to the *ts, ca*, and *att* phenotypes. The
mutations in C and F were not *ts* at 40°C but nonetheless

conferred the *att* phenotype, and they, therefore, are non-*ts*
att mutations. The HN mutation did not confer the *ca*, *ts* or
att phenotypes. Viruses possessing the 3´ leader and N
mutations were *ts*, but exhibited only marginal attenuation in
5   the lower respiratory tract of hamsters. Recombinants
possessing several combinations of mutations exhibited a
greater level of temperature sensitivity than *cp*45, but the
increased level of temperature-sensitivity was not reflected
in an increase in attenuation *in vivo*. These latter findings
10   indicate that the multiple mutations identified in *cp*45 are
interacting to affect replication *in vitro*. The presence of
multiple *ts* and non-*ts* attenuating mutations in *cp*45 likely
contributes to its high level of attenuation and phenotypic
stability. Knowledge of the phenotypes associated with the
15   various mutations of *cp*45 provided herein allows for accurate
monitoring of biologically derived PIV viruses and ready
manipulation of recombinant virus to achieve a large
assemblage of useful vaccine recombinants within the
invention.

20   **Viruses and cells.**

The rPIV3s, PIV3 JS wt and *cp*45 viruses described in
the present Example were grown in simian LLC-MK2 cells (ATCC
CCL 7.1) as described above (see also, Durbin et al., <u>Virology</u>
<u>235</u>:323-332, 1997a; Hall et al., <u>Virus Res. 22</u>(3): 173-184,
25   1992; Skiadopoulos et al., <u>J. Virol 72</u>(3):1762-8, 1998). The
modified vaccinia virus Ankara was provided as described
above. HEp-2 (ATCC CCL 23) and LLC-MK2 cells were maintained
in OptiMEM I (Life Technologies, Gaithersburg, MD)
supplemented with 2% FBS and gentamicin sulfate (50ug/mL), or
30   in EMEM supplemented with 10% FBS, gentamicin sulfate
(50ug/mL), and 2mM glutamine. L-132 cells (ATCC CCL 5) were
grown in Earl's MEM (Life Technologies) supplemented with 10%
FBS, 2mM glutamine, 20 mM Hepes, 1 mM non-essential amino
acids, and 100 units streptomycin-neomycin/ml.

35   **Construction of point mutations in PIV3.**

Subgenomic fragments of p3/7(131)2G, the antigenomic
cDNA clone of PIV3 JS wt used above to recover infectious
virus (see also, Durbin et al., <u>Virology 235</u>:323-332, 1997a;

92

Skiadopoulos et al., J. Virol. 72(3):1762-8, 1998), were cloned into pUC19 vectors modified to accept these fragments, using standard molecular cloning techniques. Point mutations corresponding to mutations identified in cp45, as well as mutations creating or ablating silent restriction enzyme recognition sequences (Table 8) were introduced using the Transformer Mutagenesis Kit (Clontech, Palo Alto, CA) as described previously.

Table 8: PIV3 cp45 mutations introduced into rPIV3

	seq id no	region affected	nt position[a]	sequence changes[b]	restriction marker[c]	amino acid substitution[d]
1	46 47	3´ leader	20	TTGTCTGGGAAT TTGCCTGGGAAT	none	non-coding
2	46 48	3´ leader	20	TTGTCTGGGAAT TTGTTTGGGAAT	none	non-coding
3	46 49	3´ leader	20	TTGTCTGGGAAT TTGTCTGGTAAT	none	non-coding
4[e]	50 51	3´ leader	40	AACTTTAAATTA AACTTAAAATTA	-Dra I	non-coding
5[e]	52 53	N gene start	60	TTAAAGACATTG TTTAAGACATTG	none	non-coding
6[e]	54 55	N	390	GCAGATGTCAAG GCAGATGCCAAG	none	Val-96 to Ala
7[e]	56 57	N	1271	CGAATCTAAAGA CGAAGCTAAAGA	none	Ser-389 to Ala
8	58 59	C	2076	GAAATATTGATC GAAACATTGATC	-Ssp I	Ile-96 to Thr
9	60 61	M	4341	TCTCTACCCAAC TCGTTAACCAAC	+Hpa I	Pro-199 to Thr
10[f]	62 63	F	6323	AGTACAATAGGT AGTACTGTGGGT	+Sca I	Ile-420 to Val
11	64 65	F	6419	GCACTTGATCCA ACACTGGATCCA	+Bam HI	Ala-450 to Thr
12	66 67	HN	7944	CCATCATTGTTGTTGACAA CCATCATTGTGGCTGACAA	+Bst XI	Val-384 to Ala
13	68 69	L	11468	TTACATGGCCA TCACATGGCGA	-Eae I	Tyr-942 to His
14	70 71	L	11618	TTTGGACTGGGC TTTTGATTGGGC	-Bsr I	Leu-992 to Phe
15[f]	72 73	L	13308	GGTCCTAATACT GGGCCTAATATC	-Ava II	Thr-1558 to Ile

a. Position of the first nucleotide in the PIV3 sequence shown. b. Wild type sequence is shown above the mutant sequence. Nucleotide changes are underlined. Codon substitutions are in bold font. c. Each relevant restriction endonuclease recognition sequence is in italics; (+) indicates addition of new restriction endonuclease recognition sequence; (-) indicates ablation of a naturally occurring restriction endonuclease recognition sequence. d. Mutations are indicated as the three letter amino acid assignment of wt, followed by the amino acid position, followed by the cp45 assignment. e. These mutations were identified by Joanne Tatem (unpublished observations), the others were from Stokes et al., Virus Res. 30(1):43-52, 1993. f. Two nucleotides were changed in the indicated codon in order to reduce the chance of reversion to wild type sequence.

After mutagenesis, restriction endonuclease
fragments were sequenced completely and cloned back into the
full-length clone, p3/7(131)2G. The 3´ leader and N mutations
were amplified by reverse transcription (RT)-PCR directly from

5   PIV3 cp45 virion RNA and were cloned into pLeft+2G (see
above), or a modified pUC19 vector for further manipulation.
Combinations of mutations were constructed using standard
molecular cloning techniques. The full-length plasmid clone
containing the cp45 mutations, designated pFLCcp45, was

10   completely sequenced to determine if extraneous mutations had
been introduced during the cloning process, but none were
found.

Recovery of recombinant PIV3 (rPIV3).

Each full-length antigenomic cDNA bearing cp45

15   mutations, together with the three support plasmids pTM(N),
pTM(P no C) and pTM(L), was transfected into HEp-2 cells on
6-well plates (Costar, Cambridge, MA) using LipofectACE (Life
Technologies, Gaithersburg, MD) and MVA-T7 as described above.
After incubation at 32°C for 4 days, the transfection harvest

20   was passaged onto LLC-MK2 cells in T-25 flasks which were
incubated at 32°C for four to eight days. This harvested
virus was called passage 1 and was subjected to three rounds
of plaque purification on LLC-MK2 cells as described above.
Each biologically-cloned recombinant virus was amplified twice

25   in LLC-MK2 cells at 32°C to produce virus for further
characterization. Virus was concentrated from clarified
medium by polyethylene glycol precipitation (see, Mbiguino and
Menezes, J. Virol Methods 31:2-3, 1991, incorporated herein by
reference in its entirety), and viral RNA (vRNA) was extracted

30   with Trizol Reagent (Life Technologies). Reverse
transcription was performed on vRNA using the Superscript II
Preamplification System (Life Technologies) with random
hexamer primers. The Advantage cDNA PCR kit (Clontech) and
sense and antisense primers specific for various portions of

35   the PIV3 genome were used to amplify fragments for restriction
endonuclease analysis. The PCR fragments were analyzed by
digestion with each of the restriction enzymes whose

recognition sites had been created or ablated during
construction of the mutations (Table 8).

Efficiency of plaque formation (EOP) of rPIV3
bearing cp45 mutations at permissive and restrictive
5   **temperatures.**

The level of temperature-sensitivity of plaque
formation *in vitro* of control and recombinant viruses was
determined at 32°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, and 41°C
in LLC-MK2 monolayer cultures as described above (see also,
10   Hall et al., <u>Virus Res. 22</u>(3):173-184, 1992, incorporated
herein by reference in its entirety). Plaques were enumerated
by hemadsorption with guinea pig red blood cells following
removal of the methylcellulose overlay, or alternatively, the
viral plaques present in the monolayer were identified by
15   immunoperoxidase staining with a mixture of two PIV3-specific
anti-HN murine mAbs 101/1 and 454/11 diluted 1:250 (see,
Murphy et al., <u>Vaccine 8</u>(5):497-502, 1990; van Wyke Coelingh
et al., <u>Virology 143</u>(2):569-582, 1985).

Evaluation of rPIV3 mutant viruses for
20   **cold-adaptation phenotype.**

Growth of mutant and wt rPIV3 viruses was determined
at 32°C and 20°C on confluent L-132 cell monolayers prepared in
24-well tissue culture plates. Duplicate wells of each of two
plates were inoculated with 0.2 ml of each mutant or wt rPIV3
25   virus at a multiplicity of infection of 0.01. After one hour
adsorption at room temperature, the inoculum was aspirated and
the monolayers were washed with 1 ml of PBS per well. The
inoculated cultures were overlaid with 0.5 ml of Earl's MEM
supplemented with 10% FBS, 2mM glutamine, 20 mM Hepes, 1 mM
30   non-essential amino acids, and 100 units
streptomycin-neomycin/ml. One plate was sealed in a
waterproof pouch (Kapak) and then submerged in a 20°C bath for
13 days. The duplicate plate was placed at 32°C in a CO_2
incubator for 3 days. At the end of the incubation period,
35   virus was harvested by freeze/thawing. The titer of virus
recovered from each well was determined by plaque assay in
LLC-MK2 cells at 32°C using hemadsorption with guinea pig red

blood cells to visualize plaques. Two wt and two *cp*45
reference stocks were used as controls.

Hamster studies.

5 week-old Golden Syrian hamsters in groups of five
were inoculated intranasally with 0.1 ml OptiMEM I per animal
containing $10^{6.0}$ pfu of JS wt rPIV3, PIV3 *cp*45 virus, or one
of the mutant rPIV3s. On day 4 post-infection, the hamsters
were sacrificed, the lungs and nasal turbinates were
harvested, and the virus was quantified as described above.
The mean $\log_{10}TCID_{50}$/gram at 32°C was calculated for each group
of hamsters.

RESULTS

Introduction of the PIV3 *cp*45 mutations into JS wt rPIV3.

The fifteen mutations in the 3ʹ leader, the N GS
signal, and the N, C, M, F, HN and L proteins of *cp*45 (Table
8) were introduced into the complete PIV3 antigenomic cDNA by
site directed mutagenesis or by direct PCR amplification of a
segment of *cp*45 cDNA bearing the desired mutations. The
following antigenomic cDNAs were made (see Fig. 13): (i) r*cp*45
3ʹN, containing the four point mutations of the leader region,
the point mutation in the N GS signal, and the two amino acid
changes in the N protein; (ii) r*cp*45 C, containing the single
amino acid change in the C protein; (iii) r*cp*45 M, containing
the single amino acid change in M, (iv) r*cp*45 F, containing
the two amino acid changes in F; (v) r*cp*45 HN, containing the
single amino acid change in HN; (vi) r*cp*45 L, containing the
three amino acid changes in L, as described above; (vii) r*cp*45
3ʹNL, containing the mutations from i. and vi. above; (viii)
r*cp*45 3ʹNCMFHN, containing all of the mutations except for the
three in L; and (ix) r*cp*45, containing all fifteen *cp*45
mutations listed in Table 8. In most cases, each *cp*45 change
was engineered to be accompanied by one or more nearby silent
changes which introduced or removed a restriction enzyme
recognition site (Table 8). These served as markers to
confirm the presence of the mutation in the cDNA and in
recovered virus. Also, two of the amino acid coding changes
(mutation numbers 10 and 15 in Table 8) were made using two

96

nucleotide changes rather than the single change found in
cp45, reducing the possibility of same-site reversion to wt.
The cp45 cDNA, which contains all fifteen of the cp45 changes
in Table 8, was assembled from the same mutagenized cDNA
5   subclones as were used to introduce cp45 changes into the
other antigenomic cDNAs; it was sequenced in its entirety, and
was confirmed to contain only the intended mutations. This
indicated that all of the regions which had been subjected to
mutagenesis or PCR and had been manipulated by cloning
10 possessed the desired sequences and lacked other unwanted
mutations. Each full-length plasmid bearing one or more of
the cp45 mutations was transfected into HEp-2 cells along with
support plasmids and MVA-T7 to produce recombinant PIV3 as
described above. Analysis of RT-PCR fragments encompassing
15 the mutations indicated in Table 8 were amplified from virion
RNA of the various recombinant viruses indicated in Figure 13
confirmed the presence of the introduced mutations, and other
unintended mutations were not found.

              Plaque morphology.
20   Several of the recombinant viruses exhibited
distinctive plaque morphologies when tested on LLC-MK2 cells.
JS wt rPIV3 plaques averaged 1mm in size, and were
indistinguishable in size from the biologically derived JS wt
PIV3. Plaques of the cp45 and rcp45 viruses were larger than
25 wt, averaging two- to three-fold larger in diameter than wt,
and were indistinguishable from each other. This demonstrated
the comparability of the biologically-derived and recombinant
cp45 virus for this phenotype.

              Efficiency of plaque formation of rPIV3s bearing the
30 **cp45 mutations in LLC-MK2 cells at permissive and restrictive**
temperatures.
              The rPIV3s were assayed for their ability to form
plaques at permissive and restrictive temperatures ranging
from 32°C to 41°C (Table 9). Analysis of the ts phenotypes of
35 viruses bearing individual components of cp45 revealed that
the rcp45 3´N and rcp45 M viruses had a shutoff temperature of
40°C, and the rcp45 C mutant had a shutoff of 41°C. The
shutoff temperature of rcp45 F and rcp45 HN mutants was

greater than 41°C. Consistent with the above results, the
rcp45 L virus had a shutoff temperature of 39°C. A virus is
considered to have a ts phenotype, for example, if its
reduction of replication at 40°C (ie. titer at 32°C minus titer
at 40°C) is approximately ≥ 100-fold that of wt virus at 40°C.
Applying this definition, the present results indicated that
at least two regions of cp45 (3´N, and L) contribute to the ts
phenotype.

Table 9: The efficiency of plaque formation (EOP) of recombinant and biologically-derived viruses at permissive and restrictive temperatures.

Virus	Mean Virus Titer[a] (log$_{10}$ pfu/ml)								ts phenotype
	32°C	35°C	36°C	37°C	38°C	39°C	40°C	41°C	
rcp45 3'N	7.1	---	---	7.0	6.4	5.4	4.2	<3.7	+
rcp45 C	6.9	---	---	7.0	6.7	6.6	5.9	<3.7	-
rcp45 M	7.7	---	---	7.4	7.0	6.5	5.3	<3.7	-
rcp45 F	7.5	---	---	7.2	6.0	6.6	5.9	5.7	-
rcp45 HN	6.4	---	---	6.5	6.2	6.4	4.7	4.4	-
rcp45 L	7.3	---	---	7.2	6.7	4.0	<0.7	<0.7	+
rcp45 3'NL	7.3	5.7	<0.7	<0.7	<0.7	<0.7	<0.7	---	+
rcp45 3'NCMFHN	7.2	5.6	<0.7	2.0	2.4	<0.7	<0.7	---	+
rcp45	8.5	7.5	7.1	6.4	6.0	2.0	<0.7	<0.7	+
cp45[b]	8.3	8.0	7.4	7.0	6.2	2.3	<0.7	<0.7	+
rPIV3 wt	7.3	7.3	7.0	7.4	7.6	7.7	6.8	6.0	-

a. Plaques were enumerated by immunoperoxidase staining after incubation for 6 days at the indicated temperature. Values are the mean of two experiments, values in bold are from a single experiment. Underlined values represent the lowest non-permissive temperature at which a 100-fold reduction of plaquing efficiency occurs compared to titer at 32°C, and this is defined as the shutoff temperature of plaque formation. b. cp45 is biologically-derived and the other viruses are recombinants. c. A virus is defined as bearing the ts phenotype if its reduction of replication at 40°C (ie. titer at 32°C minus titer at 40°C) is ≥ 100-fold that of wt virus at 40°C.

As shown in Table 9, rcp45, containing all of the cp45
mutations, had a shut-off temperature of 38°C, which was
identical to that of the biologically-derived cp45. These

5   results show that the ts phenotype of cp45 was successfully
reproduced in rcp45. In addition, these results validate the
sequence analysis of cp45 and the subsequent reconstruction of
mutations into recombinant virus.

The rcp45 3´NCMFHN virus, which is identical to

10   rcp45 except that it lacks the three L mutations, exhibited a
shutoff temperature of 36°C. Since the L mutations are known
to confer temperature-sensitivity individually and in
combination, it is somewhat paradoxical that rcp45 3´NCMFHN
was more, rather than less, ts than cp45. This implies that

15   there is an interaction of mutations within cp45 whereby
mutations compensate for each other to give a level of
temperature- sensitivity which is less than the sum of the
individual components.

Virus rcp45 3´NL was constructed to investigate

20   whether the L mutations interact with the leader and/or N
mutations, since all of these elements are believed to
interact during RNA synthesis. This virus had a shutoff
temperature 36°C , compared to 40°C and 39°C for rcp45 3´N and
rcp45 L, respectively. These results suggest that there is an

25   interaction between the 3´N and L mutations that results in
augmentation of temperature-sensitivity. These results also
provide another example in which a subset of cp45 mutations
specifies a level of temperature sensitivity greater than that
observed for rcp45 which contains the entire set of mutations

30   .

ca phenotype of rPIV3s bearing cp45 mutations.

The rPIV3s were analyzed to determine which genetic
elements of cp45 specified the ca phenotype (Table 10). It
was demonstrated above that rcp45 L is ts and att, but not ca.

35   This indicates that the genetic elements specifying the
greater part of the ca phenotype are located outside L, and
this was confirmed in the present study. Each of the rPIVs
possessing the 3´ leader and N mutations were ca except rcp45

3´NCMFHN, which exhibited an intermediate phenotype. This shows that the *ca* phenotype is specified mostly within the 3´N region. The finding that the level of *ca* of viruses containing the 3´N segment is less than that of *cp*45 or r*cp*45 indicates that other regions of *cp*45 contribute to the *ca* phenotype, even though this is not apparent from analysis of the other regions individually. The r*cp*45 3´NL virus is more *ca* than the r*cp*45 3´N virus, suggesting that the L mutations may make a modest contribution. The biologically-derived *cp*45 and r*cp*45 viruses exhibit comparable levels of *ca*, indicating that this phenotype, like the plaque size and *ts* phenotypes, was successfully reproduced in the recombinant *cp*45 virus provided herein. Therefore, the *ca* phenotype, like the *ts* phenotype, is a composite phenotype reflecting individual contributions to the overall phenotype as well as contributions from interacting genetic elements.

Table 10 :   Growth of wt and mutant PIV3s at 20°C compared to 32°C[a] (cold adaptation phenotype).

Virus	20°C	32°C	ca Phenotype
*cp*45[b]	6.72	8.49	+
r*cp*45	6.57	8.35	+
r*cp*45 3´NL	5.02	7.23	+
r*cp*45 3´N	4.39	8.53	+
r*cp*45 3´NCMFHN	3.53	8.08	+/-
r*cp*45 L	3.18	7.98	-
r*cp*45 C	2.52	8.32	-
r*cp*45 F	2.47	8.10	-
r*cp*45 HN	2.11	8.01	-
r*cp*45 M	1.76	8.21	-
PIV3 WT	2.57	8.43	-

a. Virus titer is expressed in $\log_{10}$ PFU/mL. b. *cp*45 is biologically-derived and the other viruses are recombinant. The *ca* phenotype is defined as a greater than 10-fold increase in growth at 20°C relative to wt.

Growth of the rcp45 mutant viruses in hamsters.

Groups of five Golden Syrian hamsters were inoculated intranasally with 10^6 TCID$_{50}$ of recombinant or

biologically-derived virus, and the level of virus replication
in the lungs and nasal turbinates was determined four days
later (Table 11). The fourth day post-inoculation has been
shown previously to be the peak of virus replication in

5   hamsters for both the wt and cp45 viruses (see, Crookshanks
and Belshe, _J. Med Virol 13_(3):243-9, 1984, incorporated
herein by reference in its entirety). The rcp45 virus was
reduced approximately 40-fold in replication in the nasal
turbinates and 1000-fold in the lungs, and thus was as

10   attenuated as the biologically-derived cp45 virus. These
results indicate that the attenuation phenotype of cp45 was
successfully reproduced in its recombinant version. Since
each phenotype of cp45 was fully reproduced in rcp45, the
additional five mutations in cp45 that were not included in

15   this Example likely made little contribution to the properties
of cp45.

Table 11: Level of replication in the upper and lower
respiratory tract of hamsters[a] of wt and mutant PIV3s [b].

Virus	Mean virus titer ($\log_{10}$ $TCID_{50}$/g ± S.E[c]) in:	
	Nasal turbinates	Lungs
JS wt rPIV3	6.9 ± 0.2	5.4 ± 0.5
rcp45 3´N	6.5 ± 0.2	3.9 ± 1.1
rcp45 C	4.8 ± 0.3	3.1 ± 0.7
rcp45 M[d]	6.8 ± 0.2	6.7 ± 0.3
rcp45 F	4.6 ± 0.2	3.4 ± 0.6
rcp45 HN	6.3 ± 0.2	5.3 ± 1.0
rcp45L	4.2 ± 0.1	2.1 ± 0.3
rcp45 3´NL	4.7 ± 0.2	2.1 ± 0.3
rcp45 3´NCMFHN	5.8 ± 0.3	2.9 ± 0.9
rcp45	5.3 ± 0.1	2.4 ± 0.2
cp45	4.9 ± 0.4	1.9 ± 0.2

a. Groups of five hamsters each were intranasally administered
$10^{6.0}$ $TCID_{50}$ of virus per animal in an 0.1ml inoculum, and lungs
and nasal turbinates were harvested four days later. b. cp45
is a biologically-derived virus, the other viruses are
recombinant. c. $TCID_{50}$, 50% tissue infectious dose ± Standard
Error. d. The virus pool used in this study was found to
contain a mixed plaque phenotype. The attenuation phenotype
of this mutant will be reassessed using additional virus
preparations.

        As demonstrated above, the mutations in the L gene
of cp45 specify the majority of the attenuation phenotype of
this virus. In the present Example, the contribution of the
cp45 mutations outside of L as a group was examined. The
rcp45 3´NCMFHN mutant was only slightly reduced in replication
in the nasal turbinates, but was more than 100-fold reduced in
replication in the lungs, which shows that additional
attenuating mutations were present outside of the L protein.
Importantly, if each of the three mutations in the L gene of
rcp45 reverted to wild type sequence, the resulting virus,
rcp45 3´NCMFHN, would still retain the attenuation phenotype.
The rcp45 M and rcp45 HN mutant viruses were not defective for
replication in the respiratory tract of hamsters, and the
rcp45 3´N virus showed only a marginal decrease in replication

in the lower respiratory tract. This suggests that the
mutations present in the 3´ leader, in the N gene start site
and the N , M and HN proteins are not attenuating in and of
themselves. However, these mutations could contribute to the
overall attenuation of cp45 in the context of the other cp45
mutations. Also, individual mutations within the 3´N region
may have effects which are not apparent when the set of
mutations is analyzed together, which can be readily
determined according to the present disclosure.

Replication of the rcp45 C and rcp45 F mutant
viruses was approximately 100-fold reduced in both the nasal
turbinates and the lungs, demonstrating that the mutations
present in the C and F proteins of cp45 confer the attenuation
phenotype in hamsters, although the level of attenuation is
not as great as that conferred by the cp45 L mutations. As
described above, the rcp45 F and rcp45 C mutant viruses did
not possess the ts phenotype, and therefore, the mutations
that occur in the C and F proteins are considered to be non-ts
attenuating mutations.

EXAMPLE XI
Recovery of Recombinant, Chimeric PIV3 in which the
Hemagglutinin and Fusion Glycoproteins have been Substituted
with
Corresponding Glycoproteins From PIV1

Within the present example, a chimeric rPIV virus is
generated and selected which incorporates one or more
heterologous genes or large polynucleotide sequences from one
PIV into a different rPIV background. Within this aspect of
the invention, individual genes or gene segments of one PIV
are substituted by counterpart sequence(s) from a heterologous
PIV or other source. In one embodiment described in the
present Example, tools and methods are provided for
substituting, e.g., the HN and/or F protective antigens of
HPIV1 or HPIV2 into a recombinant HPIV3 to yield a chimeric
recombinant suitable for developing live-attenuated vaccines.

Viruses and cells.

The PIV1 strain used in this study,
PIV1/Washington/20993/1964 (PIV1/Wash64), was isolated in
Washington DC in 1964 and was confirmed to be a virulent wild
type virus in clinical studies in adult human volunteers
5   (Murphy et al. Infect. Immun. 12:62-8 (1975), incorporated
herein by reference). It was propagated in LLC-MK2 cells
(ATCC CCL 7.1) in Opti-MEM I (Life Technologies) with 50 μg/ml
gentamicin sulfate, 2 mM glutamine and 0.75 μg/ml trypsin
10   (Catalog No. 3741, Worthington Biochemical Corp., Freehold,
NJ). The Greer strain of human PIV2 (Catalog No.
V-322-001-020, NIAID Repository, Rockville, MD) used in the
hemagglutination-inhibition assay (HAI) was propagated in the
same way. The JS strain of human PIV3 virus and its
15   recombinant derivative from cDNA (rPIV3/JS) with wild type
phenotype were propagated as described above. The modified
vaccinia Ankara (MVA) recombinant that expresses the
bacteriophage T7 RNA polymerase is described in Wyatt et al.,
Virology 210:202-205 (1995) (incorporated herein by
20   reference).

HEp-2 cells were obtained from ATCC (ATCC CCL 23)
and maintained in Opti-MEM I (Life Technologies) with 2% fetal
bovine serum (FBS), 50 μg/ml gentamicin sulfate and 2 mM
glutamine.
25   **Construction of a cDNA encoding a complete chimeric
PIV3-PIV1 antigenome.**

A cDNA encoding a full-length PIV3 antigenomic RNA
in which the PIV3 HN and F ORFs were replaced by their PIV1
counterparts was constructed as shown in Figure 14. cDNA
30   clones of the HN and F genes of PIV1/Wash64 were prepared from
RNA extracted from LLC-MK2 cells which had been infected with
PIV1/Wash64 wild type virus. cDNA was generated using the
SuperScript Preamplification System using random hexamer
primers (Life Technologies). PIV1 F and HN cDNAs were
35   amplified with Vent DNA polymerase (New England Biolabs,
Beverly, MA) using gene specific primer pairs based on
consensus sequences present in GenBank. All primers described
below are annotated so that PIV specific sequences are

underlined, restriction sites are in italics, nt altered from
wild type sequences are in lowercase, and start and stop
codons are in bold. The positive sense PIV1 F primer,
hybridizing to nt 69-97 upstream of the start codon, was
5'-GGGAAAGAAtCCAGAGACAAGAACGG-3' (SEQ ID NO: 33). The
negative- sense PIV1 F primer, hybridizing to nt 36-61
downstream of the F stop codon, was
5'-GGTGAAGTTGTGGATccATTTGATTG-3' (SEQ ID NO: 34). It carries
a *BamH* I site. The positive-sense primer for PIV1 HN was 5'-
CAACCTGTAAGGtAcCAGCATCCG-3'(SEQ ID NO: 35). It hybridizes to
nt 13-36 upstream the HN start codon and carries a *Kpn* I site.
The negative-sense PIV1 HN primer was
5'-GATATGGTGTTaGGcCTTGATCTGTTC-3' (SEQ ID NO: 36). It
hybridizes to the last two nt of the stop codon and 25 nt
further downstream and carries a *Stu* I site. The PIV1 F cDNA
was cloned as a *BamH* I and blunt-end fragment into *BamH* I-EcoR
V digested pLITMUS28 (New England Biolabs), while the PIV1 HN
cDNA was cloned as a *Kpn* I-*Stu* I fragment into the same
vector. The nt sequences of the resulting plasmids,
designated as pLit.1HNwt and pLit.1Fwt (GenBanK Accession
number: AF016280, AF016279), were determined using the
Circumvent Sequencing Kit (New England Biolabs). These two
clones were modified (Fig. 14) using mutagenic PCR primers to
delete their non-coding regions and to introduce new
restriction sites flanking their start and stop codons for the
purpose of constructing the PIV3-1 chimeric HN and F genes.
The sequences of positive-sense and negative-sense PIV1 F
mutagenic primers were 5'-CgccATGgAAAAATCAGAGATCCTCTTCT-3'
(SEQ ID NO: 37) and 5'-CtggatcctAATTGGAGTTGTTACCCATGTA-3' **(SEQ
ID NO: 38)**, respectively, the introduced restriction sites are
Nco I and *BamH* I. The sequences of positive-sense and
negative sense PIV1 HN mutagenic primers were 5'-
AACcATGGCTGAAAAAGGGAAAA-3' (SEQ ID NO: 39) and 5'-
GGTGAaGCTtAAGATGTGATTTTACATATTTTA-3' (SEQ ID NO: 40),
respectively, the introduced restriction sites are *Nco* I and
Hind III.
          The second step involved the modification of the
PIV3 HN and F genes to serve as acceptors of the PIV1 coding

regions generated above. The PIV3 F and HN genes were
subcloned in several steps from the full-length clone of
PIV3/JS, p3/7(131)2G to generate pLit.PIV3.HN.4 and
pLit.PIV3.F.3a (Fig. 14). The PIV3 F or HN coding regions

5   were deleted by PCR mutagenesis and replaced by restriction
sites appropriate to accept the PIV1 HN and F cDNA described
above. The sequences of the PIV3 F positive sense and
negative sense mutagenic primers were
5'-AAATA*ggatcc*CTACAGATCATTAGATATTAAAAT-3' (SEQ ID NO: 41) and

10   5'-Cgc*CATg*GTGTTCAGTGCTTGTTG-3' (SEQ ID NO: 42), respectively,
the introduced restriction sites were *Bam*H I and *Nco* I. The
sequences of PIV3 HN positive sense and negative sense
mutagenic primers were 5'-CCAC*AAgCt*TAATTAACCATAATATGCATCA-3'
(SEQ ID NO: 43) and 5'-TT*CCATgg*ATTTGGATTTGTCTATTGGGT-3' (SEQ

15   ID NO: 44), respectively, the introduced restriction sites
were *Hin*d III and *Nco* I. The stop codon for the chimeric HN
would be regenerated upon ligation with mutagenized PIV1 HN
cassette. The PIV1 HN or F cDNAs described above were
imported in as a *Nco* I-*Hin*d III fragment for HN or as a *Nco*

20   I-*Bam*H I fragment for F, which generated pLit.PIV3-1.HNhc and
pLit.PIV3-1.Fhc. These two cDNAs were then joined into
pSE.PIV3-1.hc, which was subsequently sequenced in its
entirety. Then the *BspE* I-*Sph* I fragment was inserted into
p3/7(131)2G to generate pFLC.2G+.hc (Fig. 14). The nucleotide

25   sequence of this *BspE* I-*Sph* I fragment, containing the
chimeric F and HN genes, is in GenBank (Accession No.
AF016281). Importantly, the cDNA engineering was designed so
that the final rPIV3-1 antigenome conformed to the rule of six
(Durbin et al. Virology 234:74-78 (1997), incorporated herein

30   by reference). The plasmid pFLC.2G+.hc encoding the rPIV3-1
chimera was deposited on 9/17/97 under the terms of the
Budapest Treaty with the American Type Culture Collection
(ATCC) of 12301 Parklawn Drive, Rockville, Maryland 20852,
U.S.A.

35   **Transfection.** HEp-2 cell monolayers were grown to
confluence in six-well plates (Costar Corp, Cambridge, MA) and
transfections were performed as described above. Cell
monolayers were infected with MVA-T7 at a multiplicity of

infection (MOI) of 3 in 0.8 ml of serum-free Opti-MEM I
containing 50 μg/ml gentamicin, and 2 mM glutamine and then
transfected with the three support plasmids, 0.4 μg pTM(N),
0.4 μg pTM(P), 0.05 μg pTM(L), and 5 μg the PIV3-1 antigenome
5   cDNA. The plasmids were mixed in 0.2 ml of Opti-MEM I
containing 12 μl LipofectACE (Life Technologies) and added to
each well. The PIV3/JS cDNA plasmid, p3/7(131)2G, which
encodes wild type PIV3/JS antigenomic RNA, was transfected in
parallel as a positive control. After incubation at 32°C for
10   12 hours, the transfection medium was replaced with 1.5 ml of
fresh Opti-MEM I supplemented with 50 μg/ml gentamicin, and
cultures were incubated at 32°C for two additional days.
Trypsin was added to a final concentration of 0.75 μg/ml on
day 3 post transfection. Cell culture supernatants were
15   harvested on day 4 and passaged (referred to as passage 1)
onto fresh LLC-MK2 cell monolayers. After overnight
adsorption, the medium was replaced with fresh Opti-MEM I with
0.75 μg/ml trypsin. Passage 1 cultures were incubated at 32°C
for 4 days, and the amplified virus was harvested and further
20   passaged on LLC-MK2 cells (referred to as passage 2) for
another 4 days at 32°C in the presence of 0.75 μg/ml trypsin.
The recovered virus was characterized by reaction with rabbit
PIV1 antiserum, guinea pig PIV2 antiserum, and two PIV3 HN
monoclonal antibodies in a hemagglutination-inhibition (HAI)
25   assay as described previously (van Wyke Coelingh et al.,
supra, (1985)) with the exception that chicken red blood cells
(RBCS) were used for viruses possessing the PIV1 HN
glycoproteins, whereas guinea pig RBCs were used for PIV3.
The HAI was performed to determine if rPIV3 possessed the
30   monoclonal antibody resistant mutation (MARM) that marked
virus recovered from cDNA or if rPIV3-1 possesses the PIV1 HN.
        Nucleotide sequence analysis. The chimeric cDNA
construct pSE.PIV3-1.hc was sequenced with the Circumvent
Sequencing kit (New England Biolabs) before the final assembly
35   into full-length clone pFLC.2G+.hc. For analysis of RNA, the
appropriate PIVs were amplified in T75 flasks of LLC-MK2
cells. Virus was harvested on day 5 post-infection and
concentrated by polyethylene glycol precipitation (Mbiguino et

al., _J. Virol. Methods_ 31:161-70 (1991)). Viral RNA was
extracted from the viral pellets and used in reverse
transcription (RT) with the Superscript Preamplification
System (Life Technologies). RT-PCR was performed using the
5   Advantage cDNA synthesis kit (Clontech) and PIV1 or PIV3
specific primer pairs. RT-PCR products were gel purified by
electrophoresis onto, and elusion from, strips of NA45 DEAE
nitrocellulose membrane (Schleicher & Schnuell, Keene, NH),
and were sequenced.

10   **Replication of PIVs in LLC-MK2 cells.** Plaque
enumeration on LLC-MK2 monolayers was performed as previously
described except that trypsin was added in the case of the
PIV1 and rPIV3-1 viruses (Hall et al. _Virus Res._ 22:173-84
(1992)). Briefly, serially-diluted virus was inoculated onto
15   LLC-MK2 monolayers in six-well plates; virus was adsorbed for
1 hour at 32°C; the cultures were overlaid with L-15 medium
(Life Technologies) containing 0.8% agarose with or without
added trypsin and incubated at 32°C for 6 days; and plaques
were identified by hemadsorption (HAD) with guinea pig red
20   blood cells following the removal of the agarose overlay.

Growth of the PIV viruses in tissue culture was
evaluated by infecting confluent LLC-MK2 monolayers on
twelve-well plates with virus at a MOI of 0.01. After
adsorption at 32°C for 1 hour, the inoculum was replaced with
25   1.5 ml/well Opti-MEM I supplemented with gentamicin (50 μg/ml)
and trypsin (0.75 μg/ml), and further incubated at 32°C for 6
days. At 24 hour intervals, 0.3 ml medium was removed from
each well and 0.3 ml fresh medium with trypsin was added back.
The titer of virus was determined at 32°C by hemadsorption
30   assay on LLC-MK2 cell monolayers using fluid overlay as
previously described (Hall et al. _Virus Res._ 22:173-84
(1992)), and the titers were expressed as $\log_{10}TCID_{50}$/ml.

**Replication of PIVs in the respiratory tract of
hamsters.** Golden Syrian hamsters in groups of 12 were each
35   inoculated intranasally with 0.1 ml of L-15 medium containing
10^5 pfu of rPIV3/JS, rPIV3-1, or PIV1/Wash64. On days 4 and 5
post-inoculation, six hamsters from each group were
sacrificed, and their lungs and nasal turbinates harvested,

and homogenized, and virus present in the samples was titered
on LLC-MK2 cell monolayers at 32°C. The titers were expressed
as mean $\log_{10}$ TCID$_{50}$/g for each group of six hamsters.

5   RESULTS
         Construction of a cDNA clone encoding a full-length,
chimeric PIV3-1 antigenomic RNA yielded recovery of the
Chimeric Virus rPIV3-1.
         As noted above, the final construct of the PIV3 and
10   PIV1 chimeric cDNA, in which the ORFs of the JS wild type PIV3
      HN and F glycoprotein genes were replaced by those of
      PIV1/Wash64 coding sequences (Fig. 14) encodes a PIV3-1
      chimeric antigenomic RNA of 15,516 nt, which conforms to the
      rule of six (Durbin et al., Virology 234:74-78 (1997)). The
15   pFLC.2G+.hc cDNA encoding the chimeric PIV3-1 antigenome was
      transfected onto HEp-2 cells together with the N, P and L
      support plasmids. The p3/7(131)2G cDNA encoding the JS wt
      PIV3 antigenome was transfected in parallel to generate a
      rPIV3 control parental virus. Virus was recovered from each
20   transfection following the second amplification on LLC-MK2
      cells, and studies were initiated to confirm that each
      recombinant virus was derived from cDNA.
         Recombinant viruses rPIV3-1 and rPIV3 were first
      characterized for the presence of the PIV1 or PIV3 HN
25   glycoprotein by HAI assay with serotype-specific anti-HN
      monoclonal or polyclonal antibodies. As shown in Table 12,
      rPIV3 reacted with only one of the two PIV3 mAbs as expected,
      whereas its biologically derived parent PIV3/JS reacted with
      both. This confirmed that rPIV3 contained the introduced MARM
30   mutation that marks this virus as being derived from cDNA.
      The rPIV3-1 virus reacted with antibodies specific to the PIV1
      HN glycoprotein, but not to ones specific to HN of PIV3 or
      PIV2, showing that the virus contained the PIV1 HN gene as
      expected.

Table 12.   rPIV3-1 possesses the HN glycoprotein of PIV1

Virus	Hemagglutination-inhibition titer[a] (reciprocal) of indicated monoclonal antibody or polyclonal antiserum			
	PIV1[b] antiserum	PIV2[b] antiserum	α-PIV3 mAb 423/6[c]	α-PIV3 mAb 77/5[c]
PIV1/Wash64	256	32[d]	≤50	≤50
rPIV3-1	64	≤2	≤50	≤50
rPIV3/JS	4	≤2	≤50	3,200
PIV3/JS	8	≤2	12,800	6,400
PIV2/Greer	8	512	≤50	≤50

[a]Chick red blood cells (RBC) were used in HAI assay for PIV1, PIV2, and rPIV3-1 and guinea pig RBCs were used for PIV3/JS and rPIV3/JS.

[b]PIV1 rabbit antiserum was purchased from Denka Seiken Co. Ltd., Japan (Catalog No. 410-701), and PIV2 guinea pig antiserum was obtained from NIAID repository, Rockville, MD (Catalog No. V-322-50-558).

[c]Biologically derived PIV3/JS contains epitopes recognized by both mAb 423/6 and 77/5, whereas rPIV3/JS was engineered to lack reactivity with mAb 423/6.

[d]The PIV2 antiserum had some reactivity with PIV1 virus, and therefore is not completely type specific.

It was next confirmed that the rPIV3-1 virus contained the engineered, chimeric PIV3-1 HN and F genes. As designed, the genetic structure of rPIV3-1 was unique in four junction regions when compared with either of its parents, PIV1/Wash64 or rPIV3/JS (boxed in Figure 15A). These regions are the transition points at which the sequence switches from the PIV3 non-coding region to the PIV1 coding region and then from the PIV1 coding region back to the PIV3 non-coding region. Using the primer pair A specific to PIV3 M and L genes, or primer pair B specific to the PIV1 M and the very 3'-end of HN gene, RT-PCR products were generated for virion-derived RNAs from rPIV3-1, rPIV3/JS, and PIV1/Wash64. Control reactions showed that the RT step was required for generation of RT-PCR products, indicating that an RNA template, rather than contaminating DNA, was required to produce the RT-PCR product. An early indication that rPIV3-1

111

was indeed a chimeric virus came from the finding that only
the PIV3-specific primer pair A generated the expected 4.6 kb
cDNA product that spans the F and HN genes (Fig. 15B). Thus,
while rPIV3-1 virus contains only HPIV1-specific HN

5   glycoprotein (See Table 12), the non-coding regions are
specific to PIV3. Conversely, the PIV1 specific primer pair B
amplified an appropriately sized product from PIV1 control but
not from rPIV3-1. Restriction digestion analysis also
demonstrated that rPIV3-1 RT-PCR product had unique

10   restriction patterns different from that of rPIV3/JS and
PIV1/Wash64 and appropriate for its predicted sequence.
          The nt sequence of the 4.6 kb RT-PCR product of
rPIV3-1 was determined in its four regions (Fig. 15 A) and
compared with that of rPIV3/JS and PIV1/Wash64 (Fig. 16). The

15   rPIV3-1 sequence was completely in agreement with the cDNA
from which it was derived. Examination of the sequence
alignment of the Region I-IV for the three RT-PCR products
illustrates that rPIV3-1 contains the PIV1 F and HN
glycoprotein ORFs with altered start and stop codons and

20   flanked by the 5' and 3' non-coding regions of PIV3. Examples
of sequencing ladders spanning the Region III and IV of
rPIV3-1 (Fig. 17), compared in parallel with rPIV3/JS or
PIV1/Wash64, were evaluated, and this analysis confirmed that
rPIV3-1 is a recombinant chimeric virus whose structure is

25   completely in agreement with the cDNA from which it was
generated.
          Trypsin-dependence and cytopathicity of rPIV3-1 _in_
vitro.
          PIV1, like Sendai virus but contrary to PIV3,

30   requires trypsin for cleavage of its F glycoprotein in order
to undergo multicycle replication on continuous lines of
tissue culture cells (Frank et al. _J. Clin. Microbiol._ 10:32-6
(1979)). In addition, PIV1 is a non-cytopathic virus whereas
PIV3 readily produces extensive CPE (Collins et al. In _Fields_

35   _Virology_, 3rd ed., 1:1205-43 (1996)). rPIV3-1, rPIV3 and
PIV1/Wash64 were compared on the basis of these properties.
rPIV3-1, like PIV1/Wash64, had a higher HA titer using
chicken, rather than guinea pig (Table 13), RBCs. rPIV3-1,

like its PIV1/Wash64 parent, required trypsin for efficient
replication in cultures with fluid overlay as well as for
efficient plaque formation. rPIV3-1 produced plaques at 32°C,
37°C or 40°C with similar efficiency. It is therefore evident
that rPIV3-1 possesses the F glycoprotein of the PIV1 parent
virus, and it is not temperature sensitive. On the other
hand, rPIV3-1 produced CPE, as indicated by the cell rounding
and detaching in the virus infected monolayers, almost to the
same extent as its PIV3 parent suggesting that this biological
property is a function of its PIV3 genes, which lie outside of
the HN and F coding regions. Thus, rPIV3-1 possesses
biological properties from both parents which is consistent
with the findings above demonstrating that it is a chimeric
virus. This exemplary recombinant, chimeric virus within the
invention was deposited on May 21, 1998 under the terms of the
Budapest Treaty with the American Type Culture Collection
(ATCC) of 12301 Parklawn Drive, Rockville, Maryland 20852,
U.S.A.

Table 13. Comparison of the HA titer and the infectivity and
cytopathicity   of parental and chimeric PIVs[a]

Virus	HA titer using indicated RBC		Infectious titer[b] ($Log_{10}TCID_{50}/ml$) using CPE or HAD as endpoint				PFU/ml[c] (Log_{10})	
	Chicken	Guinea Pig	CPE		HAD		Trypsin	
			Trypsin		Trypsin			
			−	+	−	+	−	+
PIV1/Wash64	16	8	≤2.5	≤2.5	4.8[d]	6.3	≤0.7[e]	5.8
rPIV3-1	64	16	≤2.5	5.8	5.5[d]	7.8	≤0.7[e]	7.1
rPIV3/JS	0	8	4.5	7.3	5.0[d]	7.5	5.0	6.2

[a]Virus stocks were grown in LLC-MK2 cells which were infected at an MOI of
   0.01 and incubated for 6 days in the presence (PIV1/Wash64, rPIV3-1)
   or absence (rPIV3/JS) of 0.75 µg/ml trypsin. The resulting virus
   stocks were assayed by the tests below in the presence or absence of
   trypsin as indicated.

[b]The $TCID_{50}$ assay was read at 6 days by direct visualization of CPE or by
   hemadsorption (HAD).

[c]Plaques were visualized by HAD after six days of incubation.

[d]The HAD of PIV3-infected monolayers was grossly apparent whereas that of
   PIV1 and rPIV3-1 was observable only under the microscope in which
   single cells with RBC adsorbed were observed.

[e]The lowest level of virus detectable was $10^{0.7}/ml$.

**Comparison of the level of replication of rPIV3-1 and its
parental viruses in LLC-MK2 cells and hamsters.**

The multicycle replication of rPIV3, rPIV3-1, and
PIV1 Wash/64 viruses was evaluated following inoculation of
LLC-MK2 tissue culture cells at a MOI of 0.01 (Fig. 18). It
can be seen that the kinetics and magnitude of replication of
the three viruses are very similar. This indicates that the
substitution of the HN and F genes of PIV1 for those of PIV3
did not attenuate the virus for replication in vitro. It was
next determined whether rPIV3-1 was attenuated in vivo,
specifically for replication in the upper and lower
respiratory tracts of hamsters (TABLE 14). The observed level
of replication of rPIV3-1 was similar to, if not slightly
higher than, either parent in the upper and lower respiratory
tract of hamsters.

Table 14. Level of replication of parental and chimeric PIVs in the upper
          and lower respiratory tract of hamsters[a]

| Virus | Virus titer (mean $\log_{10}$ TCID$_{50}$/gram±S.E.) in indicated tissue | | | |
| | Nasal Turbinates | | Lungs | |
	Day 4	Day 5	Day 4	Day 5
PIV1/Wash64	5.2±.24	5.2±.12	5.0±.31	5.0±.38
rPIV3-1	4.9±.23	6.2±.17	5.8±.15	6.0±.09
rPIV3/JS	4.5±.09	5.0±.18	5.1±.26	5.0±.32

[a]Hamsters were infected intranasally with $10^{5.0}$ TCID$_{50}$ per animal of the
   indicated virus, and lungs and nasal turbinates were removed on day 4
   or 5 after infection. The titers are means of six animals per day
   and are expressed as mean $\log_{10}$TCID$_{50}$/gram ± standard error.

          In summary, the present Example demonstrates
successful recovery of a rPIV3-1 chimeric virus in which the
ORFs of the PIV1 HN and F glycoproteins were substituted for
those of rPIV3. This chimeric virus replicated like its wild
type PIV1 and PIV3 parental viruses *in vitro* and *in vivo*,
demonstrating that the substitution of the glycoprotein ORFs
did not result in attenuation of rPIV3-1. This successful
recovery of a recombinant PIV3 which bears the HN and F
glycoproteins of PIV1 is surprising because the two viruses,
representing distinct serotypes, are not closely related.   In
particular, it is remarkable that the chimeric recombinant
grows as well as the two parents. Notably, chimeric
recombinant viruses possessing a substitution in the
glycoprotein gene have also been recovered for vesicular
stomatitis virus (VSV) (Lawson et al. Proc. Natl. Acad. Sci.
USA 92:4477-81 (1995)). Specifically, the VSV G glycoprotein
gene of Indiana serotype was replaced by that from New Jersey
serotype which share only 50% amino acid sequence identity.
In contrast to rPIV3-1, the chimeric recombinant VSV$_{I/NJ}$
replicates to only 10% the level of recombinant VSV$_I$ or
biologically derived, VSV.

          In the present Example, the HN and F glycoproteins
have 43 and 47% sequence identity, respectively, between PIV1

and PIV3. The transfer of the two glycoproteins together
would, of course, obviate glycoprotein-to-glycoprotein
incompatibility (Tanabayashi, K. and Compans, R.W. _J. Virol._
70:6112-18 (1996)). On the other hand, it is generally

5   thought that the glycoproteins interact with the M protein
(which is 63% identical between PIV1 and PIV3) through their
cytoplasmic (CT) or transmembrane (TM) domains, and that this
interaction is important in virion morphogenesis and
structure. In this regard, the degree of sequence identity

10   between the HN and F proteins of the two serotypes in the TM
and CT domains is low indeed: 30% and 22%, respectively for
the TM domain, and 9 and 11% respectively for the CT domain.
In light of this low level of sequence relatedness, we have
also pursued a parallel strategy of constructing chimeric

15   glycoproteins in which the PIV1 ectodomain of each
glycoprotein was fused to the PIV3 TM and CT domains.
Regarding possible interaction with the M protein or other
internal proteins, it might be that a conserved structure,
such as a constellation of charged amino acids, is important

20   rather than a conserved sequence. Alternatively, it might be
that interaction of the TM and CT domains of the glycoproteins
with internal proteins is not as critical as has been
previously thought. It will be possible to examine these
factors more closely using the methods and tools provided

25   herein. For example, these factors will be further elucidated
by work in progress employing the methods described above to
construct a PIV3 virus bearing HN and F of PIV2.

It was expected that rPIV3-1 would require trypsin
for efficient replication in tissue culture since this is a

30   property conferred by the PIV1 F glycoprotein, and this was
found to be the case. However, it was interesting to observe
that rPIV3-1 caused CPE that more closely resembled that of
PIV3 parent virus, indicating that a PIV3 gene(s) other than
HN or F specifies this phenotype. These roles will also be

35   further elucidated using the methods and tools provided herein
to exchange additional gene(s) between the non-cytopathic PIV1
and the cytopathic PIV3.

EXAMPLE XII

Recovery of Live-Attenuated Chimeric Recombinant PIV Encoding the Internal Proteins of PIV Type 3 and the Surface Glycoproteins of PIV Type 1

In the present Example, a derivative of rPIV3-1
carrying the three temperature-sensitive and attenuating amino
acid coding changes found in the L gene of the live-attenuated
cp45 PIV3 candidate vaccine virus, termed rPIV3-1.cp45L, is
shown to exhibit a temperature sensitive phenotype with a
shut-off temperature of 38°C, similar to that of the
recombinant rPIV3cp45L which possesses the same three
mutations. rPIV3-1.cp45L is attenuated in the respiratory
tract of hamsters to the same extent as rPIV3cp45L. Infection
of hamsters with rPIV3-1.cp45L generates a moderate level of
hemagglutination-inhibiting antibodies against wt PIV1 and
induces complete resistance to challenge with wild type PIV1.
This demonstrates that attenuated chimeric PIV according to
the invention are capable of inducing a highly effective
immune response against PIV1. This disclosure also confirms
the above described data demonstrating that the surface
glycoproteins of parainfluenza viruses are sufficient to
induce a high level of resistance to homologous virus
challenge. Unexpectedly, infection with recombinant chimeric
virus rPIV3-1.cp45L or rPIV3-1, each bearing the surface
glycoprotein genes of PIV1 and the internal genes of PIV3,
also induces a moderate level of resistance to replication of
PIV3 challenge virus. This indicates that the internal genes
of PIV3 can independently induce protective immunity against
PIV3 in rodents. Thus, a reverse genetics system for PIV3 as
disclosed herein successfully produces live attenuated PIV1
vaccine candidates that are attenuated and protective in
accepted model subjects.

Viruses and cells.

The wt PIV1 strain used in this study is
PIV1/Washington/20993/1964 (PIV1/Wash64) (see, eg., Murphy et
al., Infect. Immun. 12:62, 1975 (incorporated herein by
reference in its entirety). Chimeric rPIV3-1, recovered from
chimeric PIV3 cDNA in which the PIV3 F and HN ORFs were

replaced with those of PIV1/Wash64, as described above and in Tao et al., <u>J. Virol. 72</u>:2955, 1998 (incorporated herein by reference in its entirety). These viruses were propagated in LLC-MK2 cells (ATCC CCL 7.1) in Opti-MEM I (Life Technologies, Gaithersburg, MD) with 50 μg/ml gentamicin sulfate, and 0.75 μg/ml trypsin (Catalog No. 3741, Worthington Biochemical Corp., Freehold, NJ). Trypsin is included because the F glycoprotein of PIV1, but not that of PIV3, is dependent on exogenous trypsin for cleavage when grown in cell culture under these conditions. The wt JS strain of human PIV3 virus and its recombinant derivative from cDNA (rPIV3/JS) were propagated as described above and in Durbin et al., <u>Virology</u> <u>235</u>:323, 1997 (incorporated herein by reference in its entirety). The propagation of cp45, an attenuated derivative of wt PIV3/JS (see above; and Karron et al., <u>J. Infect. Dis.</u> <u>171</u>:1107, 1995 (incorporated herein by reference in its entirety)), and rPIV3cp45L, a recombinant PIV3 carrying the three ts mutations found in the L gene of cp45, were propagated as described above and in Skiadopoulos et al., <u>J</u> <u>Virol 72</u>:1762, 1998 (incorporated herein by reference in its entirety). The modified vaccinia Ankara (MVA) recombinant that expresses the bacteriophage T7 RNA polymerase is described in <u>Virology 210</u>: 202, 1995 (incorporated herein by reference in its entirety).

HEp-2 cells, which are used in transfection, were obtained from ATCC (ATCC CCL 23) and maintained in Opti-MEM I with 2% fetal bovine serum (FBS), 50 μg/ml gentamicin sulfate.

Introduction of L mutations into rPIV3-1 antigenomic cDNA.

The three L mutations of cp45 present in the pTM(L)942/992/1558 plasmid, described above (see also, Skiadopoulos et al., <u>J Virol 72</u>:1762, 1998, were introduced into chimeric cDNA pFLC.2G+.hc (described above; see also, Tao et al., <u>J Virol 72</u>:2955, 1998), as a 2.8 kb SphI-NheI fragment (nt 11313 to 14092 in PIV3 antigenomic cDNA) to generate the full-length pFLC.2G+.hc.cp45L bearing the PIV1 F and HN ORFs and the three cp45 L gene mutations (Fig. 19).

The specific mutations present in pTM(L)942/992/1558 are indicated in the legend to Figure 19.

Transfection. HEp-2 cell monolayers in six-well plates were grown to confluence and transfections were performed as described above (see also, Tao et al., _J Virol_ _72_:2955, 1998). Trypsin was added to a final concentration of 0.75 μg/ml on day 3 post transfection prior to harvesting on day 4. Cell culture supernatants were clarified and passaged (referred to as passage 1) onto fresh LLC-MK2 cell monolayers. After overnight adsorption, the medium was replaced with fresh Opti-MEM I with 0.75 μg/ml trypsin. Passage 1 cultures were incubated at 32°C for 4 days, and the virus present in the supernatant was harvested and passaged again under the same conditions (referred to as passage 2). Virus present in the passage 2 harvest was tested for the presence of the PIV1 HN protein by hemagglutination-inhibition (HAI) assay as described above (see also Tao et al., _J Virol 72_:2955, 1998).

Replication of PIVs in LLC-MK2 at various temperatures.

Plaque enumeration on LLC-MK2 monolayers was performed as described above, with 0.75 μg/ml trypsin added to the agarose overlay in the case of PIV1, rPIV3-1, and rPIV3-1.cp45L (see also, Tao et al., _J Virol 72_:2955, 1998). After incubation at various temperatures for 6 days, the agarose overlay was removed and plaques were identified by hemadsorption (HAD) with guinea pig erythrocytes (RBCs).

Replication of PIVs in the respiratory tract of hamsters.

Groups of five hamsters were inoculated intranasally with 0.1 ml of L15 medium containing 10^6 plaque forming units (PFU) of rPIV3/JS, rPIV3cp45L, cp45, PIV1/Wash64, rPIV3-1, or rPIV3-1.cp45L. Hamsters were sacrificed on day 4 post-infection, and their lungs and nasal turbinates were harvested and homogenized. Virus present in the tissue samples was titered on LLC-MK2 cell monolayers at 32°C as described above and in Tao et al., _J Virol 72_:2955, 1998. The titers are expressed as reciprocal mean $log_{10}TCID_{50}$/gram of tissue for each group.

Immunization and challenge studies in hamsters.

Groups of ten hamsters were immunized intranasally
with 10^6 PFU of virus per animal, as described above. Serum
was collected for HAI assay prior to infection and on day 33.
The level of HAI antibodies present in the sera of each group
of 10 hamsters was determined using PIV1/Wash64 and PIV3/JS as
antigens, and the HAI titers determined are presented as mean
$\log_2$ (see also, Tao et al., J Virol 72:2955, 1998).

Thirty-five days post-immunization, five hamsters
from each group were challenged intranasally with 10^6 PFU of
either PIV1/Wash64 or rPIV3/JS. Nasal turbinates and lungs of
these challenged hamsters were harvested four days post
challenge. Virus titers in tissue samples were determined on
LLC-MK2 monolayers as described above and in Tao et al., J
Virol 72:2955, 1998, and the titers are presented as mean
$\log_{10}TCID_{50}$/gram of tissue.

RESULTS

Recovery and characterization of the recombinant chimeric virus rPIV3-1.*cp*45L.

As noted above, the cDNA clone pFLC.2G+.hc, a
full-length antigenomic cDNA of PIV3 in which the ORFs
encoding the F and HN glycoproteins have been replaced by
those of PIV1, was modified by introduction of three amino
acid coding changes (designated 942, 992 and 1558, according
to amino acid position in the L protein) identified in the L
gene of *cp*45 and shown to be independent *ts* and attenuating
mutations (Fig. 19; see also, Skiadopoulos et al., J Virol
72:1762, 1998). Each coding change was marked by the
co-introduction of contiguous translationally silent nt
substitutions that ablate a naturally-occurring restriction
site (Fig. 19; Table 8). The final full-length plasmid
construct, pFLC.2G+.hc.*cp*45L (Fig. 19), encodes a PIV3-1
chimeric antigenomic RNA of 15516 nt in length and conforms to
the rule of six (see, Durbin et al., Virology 234:74, 1997,
incorporated herein by reference in its entirety). The
authenticity of pFLC.2G+.hc.*cp*45L was confirmed by digestion
with appropriate restriction enzymes.

120

The pFLC.2G+.hc.*cp*45L cDNA was transfected into HEp-2
cells together with the PIV3 N, P and L support plasmids and
infected with MVA-T7 as described above and in Tao et al., J_
Virol 72:2955, 1998). Virus recovered after two passages on
5   LLC-MK2 cells, termed rPIV3-1.*cp*45L, was biologically cloned
by plaque-to-plaque-to-plaque passage, and amplified virus was
analyzed to confirm that it possessed the PIV1 glycoproteins
and the three introduced mutations in L. First, the presence
of the PIV1 HN protein in rPIV3-1.*cp*45L was confirmed by
10   reactivity with PIV1 specific antibodies in HAI assay as
described above and in Tao et al., J Virol 72:2955, 1998).
The presence of the chimeric PIV3-1 HN and F genes as well as
the introduced L gene mutations in rPIV3-1.*cp*45L genomic RNA
was confirmed by restriction enzyme digestion or nucleotide
15   sequence analysis of RT-PCR products generated from virion RNA
as described above and in Tao et al., J Virol 72:2955, 1998.
These data confirmed that rPIV3-1.*cp*45L is a recombinant
chimeric virus bearing the three codon substitutions of the L
gene of *cp*45.

20   **rPIV3-1.*cp*45L is temperature sensitive.**

The three L gene mutations of *cp*45 were shown above
to confer the *ts* phenotype when introduced into *wt* PIV3 (see
also, Skiadopoulos et al., J Virol 72:1762, 1998). To
evaluate whether their presence in the chimeric virus would
25   have the same effect, the efficiency of plaque formation of
rPIV3-1.*cp*45L was determined at various temperatures. As
shown in Table 15, the three L mutations indeed conferred the
ts phenotype to the chimeric virus. The level of temperature
sensitivity specified by the *cp*45 L mutations in the
30   recombinant viruses rPIV3*cp*45L and rPIV3-1.*cp*45L was
equivalent (Table 15), indicating that the effect of the
mutations is independent of the PIV3 or PIV1 HN and F
glycoproteins. The level of temperature sensitivity of
rPIV3*cp*45L and rPIV3-1.*cp*45L was comparable to that of the
35   biologically derived *cp*45 virus, despite the fact that the
latter virus possesses mutations outside of L (see, Stokes et
al., Virus Res 30:43, 1993, incorporated herein by reference
in its entirety.

Table 15. The recombinant chimeric rPIV3-1.*cp*45L candidate
vaccine virus is temperature sensitive

Virus [a]	Virus titer [b] at indicated temperatures (log$_{10}$PFU/ml)					
	32°C	36°C	37°C	38°C	39°C	40°C
rPIV3/JS	7.4	7.2	7.2	7.2	7.2	7.2
PIV1/Wash64 [c]	7.5	7.6	7.5	7.5	7.4	7.2
rPIV3-1	7.5	7.5	7.5	7.2	6.0	6.1
PIV3*cp*45 [c]	7.4	6.9	6.8	4.7 [d]	<0.7	<0.7
rPIV3*cp*45L	7.9	7.4	7.7	5.3	1.2	<0.7
rPIV3-1.*cp*45L	8.1	8.0	8.2	6.1	<0.7	<0.7

[a] Virus nomenclature: rPIV3/JS, recombinant wt PIV3 strain JS;
PIV1/Wash64, biologically-derived wt PIV1; rPIV3-1,
recombinant chimeric PIV3 in which the F and HN ORFs have been
replaced with those of PIV1/Wash64; PIV3*cp*45, biologically-
derived *cp*45 candidate vaccine virus; rPIV3cp45L, recombinant
PIV3 containing the three L gene mutations of *cp*45; rPIV3-
1.*cp*45L, recombinant chimeric rPIV3-1 containing the three L
gene mutations of *cp*45.

[b] Virus titers were determined using LLC-MK2 monolayers in 12-
well plates. Titers are the average of two assays.

[c] Biologically-derived viruses. All others are recombinant
viruses.

[d] The shut-off temperature, *i.e.* the lowest restrictive
temperature at which a two log$_{10}$ reduction in virus titer is
observed, of each *ts* virus is indicated in bold.

Level of replication of rPIV3-1.*cp*45L in hamsters.

The three L gene mutations of *cp*45 were shown above
to confer attenuation of virus replication in the upper and
lower respiratory tract of hamsters when introduced into *wt*
PIV3 (see also, Skiadopoulos et al., J Virol 72:1762, 1998).
Their effect on the chimeric virus was evaluated by intranasal
infection of hamsters, as shown in Table 16. These findings
indicate that rPIV3-1.*cp*45L indeed was attenuated at both
sites and, furthermore, that its level of attenuation was
comparable to that of rPIV3*cp*45L. Thus, the ability of the
*cp*45 L mutations to confer attenuation, like temperature
sensitivity, is independent of the antigenic specificity of
the surface glycoproteins.

Table 16. The recombinant chimeric rPIV3-1.cp45L candidate
vaccine virus is attenuated in the respiratory tract of
hamsters [a]

Virus	Virus titer in indicated tissue $(\log_{10}\text{TCID}_{50}/g\pm S.E)$ [b]	
	Nasal turbinates	Lungs
rPIV3-1.cp45L	4.6±0.3	1.9±0.4
rPIV3-1	6.0±0.3	6.3±0.4
rPIV3cp45L	3.0±0.3	<1.2
rPIV3/JS	5.7±0.3	5.0±0.3

[a] Groups of five hamsters were infected intranasally with
indicated viruses at a dosage of 10^6 PFU per hamster. On
day 4 post infection, the tissue samples were harvested and
assayed for virus.

[b] Virus titers are given as $\log_{10}\text{TCID}_{50}$ per gram of tissue.

**Infection with rPIV3-1 or rPIV3-1.cp45L, containing
the internal proteins of PIV3 and the glycoproteins of PIV1,
confers resistance to PIV1 challenge in hamsters.**

The chimeric rPIV3-1 virus and its attenuated
rPIV3-1.cp45L derivative were evaluated for immunogenicity
and protective efficacy in hamsters. As shown in Table 17,
infection with either virus induced HAI antibodies against
PIV1, but not PIV3, confirming that these chimeric viruses
possess the PIV1 HN glycoprotein and are highly immunogenic.
The level of HAI antibodies induced by rPIV3-1.cp45L was
two-fold less than that by rPIV3-1, which indicates that its
attenuation resulted in a modest decrease in immunogenicity.
Similarly, rPIV3 and rPIV3cp45L induced HAI antibodies
against PIV3, but not PIV1, and the level induced by the
attenuated virus was approximately two-fold lower. Despite
the restricted replication in hamsters of the recombinant
viruses bearing the cp45 L mutations, infection with either
rPIV3cp45L or rPIV3-1.cp45L induced complete resistance to
replication of challenge virus bearing homologous
glycoproteins.

Table 17. The recombinant chimeric rPIV3-1.cp45L candidate vaccine virus induces complete resistance to PIV1 and partial resistance to PIV3 upon challenge in hamsters [a]

Virus used for immunization	Origin of glycoproteins	Post-immunization HAI titer ($\log_2$Reciprocal±S.E.)		Virus Titer in Indicated Tissue [c] ($\log_{10}$TCID$_{50}$/Gram±S.E.)			
				Replication of PIV1 challenge virus		Replication of PIV3 challenge virus	
		α-PIV1	α-PIV3	Nasal Turbinates	Lungs	Nasal Turbinates	Lungs
Control [b]	-	≤1	≤1	5.0±0.3	4.6±0.5	5.5±0.4	5.0±0.7
rPIV3-1.cp45L	PIV1	6.9±0.5	≤1	≤1.2	≤1.2	1.9±0.6	1.7±0.5
rPIV3-1	PIV1	7.9±0.4	≤1	≤1.2	≤1.2	2.9±0.3	2.6±0.4
rPIV3cp45L	PIV3	≤1	9.3±0.2	4.6±0.2	2.8±0.7	≤1.2	≤1.2
rPIV3/JS	PIV3	≤1	10.3±0.3	4.6±0.4	2.4±0.5	≤1.2	≤1.2
PIV3cp45 [d]	PIV3	≤1	8.9±0.4	4.9±0.2	2.5±0.8	≤1.2	≤1.2

[a] Groups of 10 hamsters were immunized intranasally with 10^6 PFU of indicated viruses. Post-immunization sera were collected on days 33, two days prior to challenge (see [c]).

[b] Hamsters in control group were not inoculated.

[c] Five weeks after immunization, five hamsters from each group were challenged intranasally with 10^6 PFU of indicated virus. Tissue samples were harvested 4 days post challenge. Viruses present in tissue samples were titered on LLC-MK2 monolayers and the data are presented as $\log_{10}$TCID$_{50}$/gram of tissue ± standard error.

[d] Biologically-derived virus.

Infection with rPIV3-1.cp45L also confers resistance to PIV3.

Information on the role of the non-HN or F glycoproteins of PIVs (i.e., the internal proteins) in resistance is limited. The disclosure and use of rPIV3-1 and rPIV3-1.cp45L herein provides an opportunity to examine the role that internal proteins play in resistance to challenge with PIV3, since the only genes shared by immunizing and challenge viruses are the internal protein genes. PIV3 challenge virus replication was significantly restricted in both the upper and lower respiratory tracts by prior infection of hamsters with rPIV3-1 or rPIV3-1.cp45L (Table 17). Thus, these data indicate that the internal proteins of PIV3, like the HN and F proteins, are capable of inducing partial resistance to replication of the challenge PIV3.

Among the findings demonstrated by the immunogenicity and efficacy studies above a particularly unexpected finding was that infection with wt or attenuated rPIV3 induced a 100-fold reduction in the replication of PIV1 challenge virus in the lungs. Thus, infection with one serotype of PIV provided significant protection against a heterologous serotype. This was unexpected in part because previous studies indicated that infection of animals with one type of human PIV did not induce significant heterologous protection against a PIV belonging to a different human serotype, conforming to a general belief that immunity to human PIV infections was largely type-specific (see, eg., Cook et al., Amer. Jour. Hyg. 77:150, 1963; Ray et al., J. Infect. Dis. 162:746, 1990, each incorporated herein by reference in its entirety).

The present Example demonstrates successful exploitation of novel methods and reagents developed for generating PIV3 vaccines to further provide rapid, rational development of live attenuated candidate vaccines for PIV1. A cDNA encoding infectious PIV3 was modified by substitution of the ORFs encoding the PIV1 HN and F protective antigens for their PIV3 counterparts. Subsequently, attenuating mutations, exemplified by three attenuating mutations present in the L

gene of the *cp*45 PIV3, were incorporated within this modified chimeric PIV3-PIV1 cDNA. From this cDNA, a recombinant virus was recovered bearing the HN and F genes of PIV1, the internal proteins of PIV3, and the PIV3 *cp*45 L gene mutations. This recombinant, rPIV3-1.*cp*45L, was temperature sensitive, highly attenuated in hamsters, and highly efficacious against PIV1 challenge in hamsters. The level of temperature sensitivity, attenuation, and immunogenicity exhibited by rPIV3-1.*cp*45L was comparable to that of *cp*45 PIV3, indicating that the phenotypes specified by the set of *cp*45 L gene mutations are independent of the HN and F surface glycoproteins. These findings, which represent the first live attenuated PIV1 vaccine candidate generated by reverse genetics, provide a generally successful scheme for developing vaccines against PIV1.

Little information is known concerning the role that internal proteins of parainfluenza viruses play in resistance to reinfection with homologous virus. Infection with vaccinia recombinants expressing N, epitopes within N, or M reportedly induce resistance to replication of challenge virus, but the magnitude of the resistance reported is less than that induced by vaccinia recombinants bearing HN or F glycoproteins (see, eg., Kast et al., *Proc. Natl. Acad. Sci. USA* 88:2283, 1991; Sakaguchi et al., *J. Gen. Virol.* 74:479, 1993; Thomson et al., *J. Immunol.* 157:822, 1996, each incorporated herein by reference in its entirety). These studies suggested that the internal proteins were making only minor contributions to resistance to reinfection. Therefore, the present disclosure presents unexpected results by showing that prior infection of hamsters with rPIV3-1.*cp*45L or rPIV3-1 induced about 250-to 4000-fold reduction of replication of PIV3 in both the nasal turbinates and lungs. These two chimeric recombinant viruses differ from the PIV3 challenge virus in that they possess the HN and F glycoproteins of PIV1 rather than PIV3, but they share all other genes with the challenge virus. The HN and F glycoproteins of PIV1 share 47% and 43% sequence identity with those of PIV3, respectively. Although it is likely that the shared internal proteins are mediating the observed

126

resistance, it is also possible that shared protein sequences
between PIV1 and PIV3 F and HN glycoproteins are contributing
to the observed immunity. For example, there are 5 stretches
in HN and 2 stretches in F extending at least 9 amino acid
residues in length that are shared between PIV1 and PIV3 and
have the potential to act as protective CTL epitopes. It is
reasonable to consider that the shared internal proteins are
contributing to the restriction of replication of *wt* PIV3
challenge virus, since this level of cross-immunity has not
been seen in previous studies (see, eg., Cook et al., Amer.
Jour. Hyg. 77:150, 1963; Ray et al., J. Infect. Dis. 162:746,
1990, incorporated herein by reference in its entirety).

The finding that the internal PIV3 proteins of the
rPIV3-1 and rPIV3-1.*cp*45L chimeras conferred resistance to
PIV3 challenge demonstrates that attenuated derivatives of
PIV3 can be used as vectors for PIV1 and PIV2 protective
antigens. Following the teachings of the invention,
immunization with one PIV3-based live-attenuated vaccine virus
can restrict the replication of other PIV3-based vaccine
viruses administered subsequently, thereby decreasing the
immunogenicity of the second virus. Since PIV3, like RSV,
induces significant illness in early infancy, a combined
RSV-PIV3 vaccine for use in the very young 2- to 4-week old
infant is therefore an important aspect of the invention (see,
eg., Collins et al., Fields Virology 3rd ed. Philadelphia:
Lippincott-Raven Publishers, 1205(1), 1996; Reed et al., J.
Infect. Dis. 175:807, 1997, each incorporated herein by
reference in its entirety). According to this aspect of the
invention, immunization with a PIV1-PIV2 vaccine will be
preferably initiated at about 6 months of age, since most PIV1
and PIV2 disease occurs after the age of six months. In the
possible circumstance that immunization with rPIV3 *cp*45
significantly inhibits replication of a chimeric recombinant
PIV3-1 vaccine virus with which it shares internal protein
genes, successful immunization with a recombinant PIV3-1
vaccine may be compromised. In this event, a trivalent PIV
vaccine will be administered simultaneously rather than
sequentially, thereby preventing the above noted inhibition.

The disclosure herein that infection with a vaccine
or *wt* PIV3 would induce a 100-fold reduction of pulmonary
virus replication of the heterologous *wt* PIV1 was clearly
unexpected, in part because the human PIV viruses are
serologically distinct by neutralization assay, and previous
studies in hamsters found that prior infection with one type
of PIV failed to induce resistance to challenge with a high
dose of a different PIV type (see eg., Cook et al., <u>Amer.
Jour. Hyg. 77</u>:150, 1963; Ray et al., <u>J. Infect. Dis. 162</u>:746,
1990; Cook et al., <u>Amer. Jour. Hyg. 69</u>:250, 1959).
Furthermore, there is little epidemiological data documenting
that prior infection with one PIV significantly modifies
subsequent infection with a heterotypic PIV.

In summary, the present Example shows that rPIV3 was
successfully converted into a vaccine for PIV1 by substituting
the ORFs encoding the F and HN glycoproteins and introducing
known attenuating mutations into the PIV3 internal genes.
Thus, the extensive methods and reagents provided herein can
be applied directly and predictably to attenuating the PIV3
backbone of the rPIV3-1 chimeric virus, as well as for
generating live-attenuated PIV2 vaccine viruses.

The foregoing disclosure makes it possible to exploit
the reagents and methods provided herein to develop a broad
assemblage of PIV and related vaccines. In this context,
recovery of live, immunogenic chimeras between PIV3 and PIV2
exemplifies powerful new tools for developing a range of
recombinant PIV viruses for vaccine use. In conjunction with
this work, identification and characterization of the genetic
basis for attenuation of naturally occurring PIV mutants,
e.g., *cp*45 and BPIV3 vaccine candidates, following the
teachings of the present disclosure also enables development
of a large host of recombinant vaccine viruses and subviral
particles. In particular, desired mutations present in
biologically derived mutant viruses will be readily identified
and selected by their introduction, singly and in combination,
into a wild type, partially attenuated, or chimeric PIV
background, as shown in the Examples above. These findings
will expand the menu of exemplary, attenuating mutations

128

within the invention which can introduced into PIV clones to
calibrate the level of attenuation and immunogenicity in
vaccine recombinants. Biologically derived mutations can also
be introduced within PIV clones having different types of
mutations, e.g., mutations involving alterations, deletions,
or substitutions of a gene or gene segment. Within this
aspect of the invention, recombinant PIV are provided which
have a selected gene deletion, addition, or substitution, such
as rPIV having a deletion of the C, D or V ORF(s). Such
alternatively mutated clones can be further modified according
to the present disclosure by introducing one or more mutations
specifying a *ts, ca* or *att* phenotype adopted from a
biologically derived mutant PIV, as exemplified by the PIV
recombinants r942, r992, r1558, r942/992, r992/1558, or
r942/1558, and r942/992/1558. In additional aspects of the
invention, biologically derived mutations will be combined
with *de novo* attenuating mutations not found in nature, as
exemplified by attenuating gene deletions, e.g., of the C, D
and/or V ORFs. Other types of mutations disclosed herein
conferring desired phenotypic characteristics will also be
combined with biologically derived, attenuating mutations,
similar to the range of combinatorial mutations disclosed for
recombinant RSV vaccine strains in United States Patent
Application Serial No. 08/892,403, filed July 15, 1997
(incorporated herein by reference). Comparable mutations can
be readily introduced, e.g., into a chimeric virus, to achieve
a desired levels of attenuation and immunogenicity in a
chimeric vaccine strain. In this manner, a large menu of
mutations are provided within the invention that are useful to
engineer a wide assemblage of live attenuated rPIV vaccines
having a desired balance of attenuation and immunogenicity,
along with other desired phenotypic characteristics.

        Although the foregoing invention has been described
in some detail by way of illustration and example for purposes
of clarity of understanding, it will be obvious that certain
changes and modifications may be practiced within the scope of
the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT:
        (A) NAME: The Government of the United States
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        (C) CITY: Bethesda
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        (F) POSTAL CODE (ZIP): 20892
        (G) TELEPHONE:
        (H) TELEFAX:
        (I) TELEX:

    (ii) TITLE OF INVENTION: PRODUCTION OF ATTENUATED PARAINFLUENZA
          VIRUS VACCINES FROM CLONED NUCLEOTIDE SEQUENCE

    (iii) NUMBER OF SEQUENCES: 74

    (iv) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

    (v) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER: WO
        (B) FILING DATE: 22-MAY-1998
        (C) CLASSIFICATION:

    (vi) PRIOR APPLICATION DATA:
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        (B) FILING DATE: 19-SEP-1997

    (vi) PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: US 60/047,575
        (B) FILING DATE: 23-MAY-1997

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(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 15669 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCTGGGTACC GGGCCCGTCG ACGCGTATAT AGTTCCTCCT TTCAGCAAAA AACCCCTCAA   60

GACCCGTTTA GAGGCCCCAA GGGGTTATGC TACTGCAGGC TCTCCCTTAG CCATCCGAGT   120

130

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GGACGTGCGT CCTCCTTCGG ATGCCCAGGT CGGACCGCGA GGAGGTGGAG ATGCCATGCC    180

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AGGATTAAAG ACATTGACTA GAAGGTCAAG AAAAGGGAAC TCTATAATTT CAAAAATGTT    300

GAGCCTATTT GATACATTTA ATGCACGTAG GCAAGAAAAC ATAACAAAAT CAGCCGGTGG    360

AGCTATCATT CCTGGACAGA AAAATACTGT CTCTATATTC GCCCTTGGAC CGACAATAAC    420

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GATTGAGAAA GATCTAAAAC GGCAAAAGTA TGGAGGATTT GTGGTTAAGA CGAGAGAGAT    660

GATATATGAA AAGACAACTG ATTGGATATT TGGAAGTGAC CTGGATTATG ATCAGGAAAC    720

TATGTTGCAG AACGGCAGGA ACAATTCAAC AATTGAAGAC CTTGTCCACA CATTTGGGTA    780

TCCATCATGT TTAGGAGCTC TTATAATACA GATCTGGATA GTTCTGGTCA AAGCTATCAC    840

TAGTATCTCA GGGTTAAGAA AAGGCTTTTT CACCCGATTG GAAGCTTTCA GACAAGATGG    900

AACAGTGCAG GCAGGGCTGG TATTGAGCGG TGACACAGTG GATCAGATTG GGTCAATCAT    960

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TTTGACTCTA TCCACTCTCA GACCAGATAT CAATAGATTA AAAGCTTTGA TGGAACTGTA   1200

TTTATCAAAG GGACCACGCG CTCCTTTCAT CTGTATCCTC AGAGATCCTA TACATGGTGA   1260

GTTCGCACCA GGCAACTATC CTGCCATATG GAGCTATGCA ATGGGGGTGG CAGTTGTACA   1320

AAATAGAGCC ATGCAACAGT ATGTGACGGG AAGATCATAT CTAGACATTG ATATGTTCCA   1380

GCTAGGACAA GCAGTAGCAC GTGATGCCGA AGCTCAAATG AGCTCAACAC TGGAAGATGA   1440

ACTTGGAGTG ACACACGAAT CTAAAGAAAG CTTGAAGAGA CATATAAGGA ACATAAACAG   1500

TTCAGAGACA TCTTTCCACA AACCGACAGG TGGATCAGCC ATAGAGATGG CAATAGATGA   1560

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CAACGACAAG AAGAAACAAA GCAGTCAACC ACCCACTAAT CCCACAAACA GAACAAACCA   1800

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AGGGTGGTAA ATTTAGAGTC TGCTTGAAAC TCAATCAATA GAGAGTTGAT GGAAAGCGAT   1980

GCTAAAAACT ATCAAATCAT GGATTCTTGG GAAGAGGAAT CAAGAGATAA ATCAACTAAT   2040

ATCTCCTCGG CCCTCAACAT CATTGAATTC ATACTCAGCA CCGACCCCCA AGAAGACTTA   2100

TCGGAAAACG ACACAATCAA CACAAGAACC CAGCAACTCA GTGCCACCAT CTGTCAACCA   2160

GAAATCAAAC CAACAGAAAC AAGTGAGAAA GATAGTGGAT CAACTGACAA AAATAGACAG   2220
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TCTGGGTCAT CACACGAATG TACAACAGAA GCAAAAGATA GAAATATTGA TCAGGAAACT   2280

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GATCTCAATG AAATTAGAAA GATGGATAAG GACTCTATTG AGGGGAAAAT GCGACAATCT   2460

GCAAATGTTC CAAGCGAGAT ATCAGGAAGT GATGACATAT TTACAACAGA ACAAAGTAGA   2520

AACAGTGATC ATGGAAGAAG CCTGGAATCT ATCAGTACAC CTGATACAAG ATCAATAAGT   2580

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ATCGACAACA ACACCGACCG GAACGAACAG ACAAGCACAA CTCCTCCAAC AACAACTTCC   2940

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AAGACAAATG GAAAGGAAAG GAAGGATACA GAAGAGAGCA ATCGATTTAC AGAGAGGGCA   3060

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ATTACAATC                                                            15669
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(2) INFORMATION FOR SEQ ID NO:2:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 31 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: cDNA

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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AATACGACTC ACTATAACCA AACAAGAGAA C                                        31
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(2) INFORMATION FOR SEQ ID NO:3:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 24 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: cDNA

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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CCAAGTACTA TGAGATGCTT CATT                                               24
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(2) INFORMATION FOR SEQ ID NO:4:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 31 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCCTATAATT TCAACATGTT GAGCCTATTT G   31

(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 31 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GATTAAAATG TTGGTCGACT TAGTTGCTTC C   31

(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 38 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCATAGAGAG TCCATGGAAA GCGACGCTAA AAACTATC   38

(2) INFORMATION FOR SEQ ID NO:7:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 37 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CGGTGTCGTT TCTTTGTCGA CTCATTGGCA ATTGTTG   37

(2) INFORMATION FOR SEQ ID NO:8:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 42 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

139

GCAAAGCGTG CCCGGGCCAT GGACACTGAA TCTAACAATG GC   42

(2) INFORMATION FOR SEQ ID NO:9:

      (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 28 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: cDNA

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GAAATTCCTT AATCGATTCT CTAGATTC   28

(2) INFORMATION FOR SEQ ID NO:10:

      (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 30 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: cDNA

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CCCATCAACT GTAACATACG TAAGAAAGAC   30

(2) INFORMATION FOR SEQ ID NO:11:

      (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 29 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: cDNA

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GGTTAGGATA TGTCGACATT GTATTTATG   29

(2) INFORMATION FOR SEQ ID NO:12:

      (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 43 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: cDNA

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GGGGTTATGC TACTGCAGGC TTTTTTTCTC CCTTAGCCAT CCG   43

(2) INFORMATION FOR SEQ ID NO:13:

      (i) SEQUENCE CHARACTERISTICS:

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        (A) LENGTH: 35 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA


    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CTCCATTCTA GANTTATAAA AATTATAGAG TTCCC                                35

(2) INFORMATION FOR SEQ ID NO:14:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 15660 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA


    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TAATACGACT CACTATAACC AAACAAGAGA AGAAACTTGT CTGGGAATAT AAATTTAACT      60

TTAAATTAAC TTAGGATTAA AGACATTGAC TAGAAGGTCA AGAAAAGGGA ACTCTATAAT     120

TTCAAAAATG TTGAGCCTAT TTGATACATT TAATGCACGT AGGCAAGAAA ACATAACAAA     180

ATCAGCCGGT GGAGCTATCA TTCCTGGACA GAAAAATACT GTCTCTATAT TCGCCCTTGG     240

ACCGACAATA ACTGATGATA ATGAGAAAAT GACATTAGCT CTTCTATTTC TATCTCATTC     300

ACTAGATAAT GAGAAACAAC ATGCACAAAG GGCAGGGTTC TTGGTGTCTT TATTGTCAAT     360

GGCTTATGCC AATCCAGAGC TCTACCTAAC AACAAATGGA AGTAATGCAG ATGTCAAGTA     420

TGTCATATAC ATGATTGAGA AAGATCTAAA ACGGCAAAAG TATGGAGGAT TTGTGGTTAA     480

GACGAGAGAG ATGATATATG AAAAGACAAC TGATTGGATA TTTGGAAGTG ACCTGGATTA     540

TGATCAGGAA ACTATGTTGC AGAACGGCAG GAACAATTCA ACAATTGAAG ACCTTGTCCA     600

CACATTTGGG TATCCATCAT GTTTAGGAGC TCTTATAATA CAGATCTGGA TAGTTCTGGT     660

CAAAGCTATC ACTAGTATCT CAGGGTTAAG AAAAGGCTTT TTCACCCGAT TGGAAGCTTT     720

CAGACAAGAT GGAACAGTGC AGGCAGGGCT GGTATTGAGC GGTGACACAG TGGATCAGAT     780

TGGGTCAATC ATGCGGTCTC AACAGAGCTT GGTAACTCTT ATGGTTGAAA CATTAATAAC     840

AATGAATACC AGCAGAAATG ACCTCACAAC CATAGAAAAG AATATACAAA TTGTTGGCAA     900

CTACATAAGA GATGCAGGTC TCGCTTCATT CTTCAATACA ATCAGATATG GAATTGAGAC     960

CAGAATGGCA GCTTTGACTC TATCCACTCT CAGACCAGAT ATCAATAGAT TAAAAGCTTT    1020

GATGGAACTG TATTTATCAA AGGGACCACG CGCTCCTTTC ATCTGTATCC TCAGAGATCC    1080

TATACATGGT GAGTTCGCAC CAGGCAACTA TCCTGCCATA TGGAGCTATG CAATGGGGGT    1140

GGCAGTTGTA CAAAATAGAG CCATGCAACA GTATGTGACG GGAAGATCAT ATCTAGACAT    1200

TGATATGTTC CAGCTAGGAC AAGCAGTAGC ACGTGATGCC GAAGCTCAAA TGAGCTCAAC    1260
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ACTGGAAGAT  GAACTTGGAG  TGACACACGA  ATCTAAAGAA  AGCTTGAAGA  GACATATAAG    1320

GAACATAAAC  AGTTCAGAGA  CATCTTTCCA  CAAACCGACA  GGTGGATCAG  CCATAGAGAT    1380

GGCAATAGAT  GAAGAGCCAG  AACAATTCGA  ACATAGAGCA  GATCAAGAAC  AAAATGGAGA    1440

ACCTCAATCA  TCCATAATTC  AATATGCCTG  GGCAGAAGGA  AATAGAAGCG  ATGATCAGAC    1500

TGAGCAAGCT  ACAGAATCTG  ACAATATCAA  GACCGAACAA  CAAAACATCA  GAGACAGACT    1560

AAACAAGAGA  CTCAACGACA  AGAAGAAACA  AAGCAGTCAA  CCACCCACTA  ATCCCACAAA    1620

CAGAACAAAC  CAGGACGAAA  TAGATGATCT  GTTTAACGCA  TTTGGAAGCA  ACTAATCGAA    1680

TCAACATTTT  AATCTAAATC  AATAATAAAT  AAGAAAAACT  TAGGATTAAA  GAATCCTATC    1740

ATACCGGAAT  ATAGGGTGGT  AAATTTAGAG  TCTGCTTGAA  ACTCAATCAA  TAGAGAGTTG    1800

ATGGAAAGCG  ATGCTAAAAA  CTATCAAATC  ATGGATTCTT  GGGAAGAGGA  ATCAAGAGAT    1860

AAATCAACTA  ATATCTCCTC  GGCCCTCAAC  ATCATTGAAT  TCATACTCAG  CACCGACCCC    1920

CAAGAAGACT  TATCGGAAAA  CGACACAATC  AACACAAGAA  CCCAGCAACT  CAGTGCCACC    1980

ATCTGTCAAC  CAGAAATCAA  ACCAACAGAA  ACAAGTGAGA  AAGATAGTGG  ATCAACTGAC    2040

AAAAATAGAC  AGTCTGGGTC  ATCACACGAA  TGTACAACAG  AAGCAAAAGA  TAGAAATATT    2100

GATCAGGAAA  CTGTACAGAG  AGGACCTGGG  AGAAGAAGCA  GCTCAGATAG  TAGAGCTGAG    2160

ACTGTGGTCT  CTGGAGGAAT  CCCCAGAAGC  ATCACAGATT  CTAAAAATGG  AACCCAAAAC    2220

ACGGAGGATA  TTGATCTCAA  TGAAATTAGA  AAGATGGATA  AGGACTCTAT  TGAGGGGAAA    2280

ATGCGACAAT  CTGCAAATGT  TCCAAGCGAG  ATATCAGGAA  GTGATGACAT  ATTTACAACA    2340

GAACAAAGTA  GAAACAGTGA  TCATGGAAGA  AGCCTGGAAT  CTATCAGTAC  ACCTGATACA    2400

AGATCAATAA  GTGTTGTTAC  TGCTGCAACA  CCAGATGATG  AAGAAGAAAT  ACTAATGAAA    2460

AATAGTAGGA  CAAAGAAAAG  TTCTTCAACA  CATCAAGAAG  ATGACAAAAG  AATTAAAAAA    2520

GGGGGAAAAG  GGAAAGACTG  GTTTAAGAAA  TCAAAAGATA  CCGACAACCA  GATACCAACA    2580

TCAGACTACA  GATCCACATC  AAAAGGGCAG  AAGAAAATCT  CAAAGACAAC  AACCACCAAC    2640

ACCGACACAA  AGGGGCAAAC  AGAAATACAG  ACAGAATCAT  CAGAAACACA  ATCCTCATCA    2700

TGGAATCTCA  TCATCGACAA  CAACACCGAC  CGGAACGAAC  AGACAAGCAC  AACTCCTCCA    2760

ACAACAACTT  CCAGATCAAC  TTATACAAAA  GAATCGATCC  GAACAAACTC  TGAATCCAAA    2820

CCCAAGACAC  AAAAGACAAA  TGGAAAGGAA  AGGAAGGATA  CAGAAGAGAG  CAATCGATTT    2880

ACAGAGAGGG  CAATTACTCT  ATTGCAGAAT  CTTGGTGTAA  TTCAATCCAC  ATCAAAACTA    2940

GATTTATATC  AAGACAAACG  AGTTGTATGT  GTAGCAAATG  TACTAAACAA  TGTAGATACT    3000

GCATCAAAGA  TAGATTTCCT  GGCAGGATTA  GTCATAGGGG  TTTCAATGGA  CAACGACACA    3060

AAATTAACAC  AGATACAAAA  TGAAATGCTA  AACCTCAAAG  CAGATCTAAA  GAAAATGGAC    3120

GAATCACATA  GAAGATTGAT  AGAAAATCAA  AGAGAACAAC  TGTCATTGAT  CACGTCACTA    3180

ATTTCAAATC  TCAAAATTAT  GACTGAGAGA  GGAGGAAAGA  AAGACCAAAA  TGAATCCAAT    3240

GAGAGAGTAT  CCATGATCAA  AACAAAATTG  AAAGAAGAAA  AGATCAAGAA  GACCAGGTTT    3300

GACCCACTTA  TGGAGGCACA  AGGCATTGAC  AAGAATATAC  CCGATCTATA  TCGACATGCA    3360
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142

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GGAGATACAC TAGAGAACGA TGTACAAGTT AAATCAGAGA TATTAAGTTC ATACAATGAG    3420

TCAAATGCAA CAAGACTAAT ACCCAAAAAA GTGAGCAGTA CAATGAGATC ACTAGTTGCA    3480

GTCATCAACA ACAGCAATCT CTCACAAAGC ACAAACAAT CATACATAAA CGAACTCAAA     3540

CGTTGCAAAA ATGATGAAGA AGTATCTGAA TTAATGGACA TGTTCAATGA AGATGTCAAC    3600

AATTGCCAAT GATCCAACAA AGAAACGACA CCGAACAAAC AGACAAGAAA CAACAGTAGA    3660

TCAAAACCTG TCAACACACA CAAAATCAAG CAGAATGAAA CAACAGATAT CAATCAATAT    3720

ACAAATAAGA AAAACTTAGG ATTAAAGAAT AAATTAATCC TTGTCCAAAA TGAGTATAAC    3780

TAACTCTGCA ATATACACAT TCCCAGAATC ATCATTCTCT GAAAATGGTC ATATAGAACC    3840

ATTACCACTC AAAGTCAATG AACAGAGGAA AGCAGTACCC CACATTAGAG TTGCCAAGAT    3900

CGGAAATCCA CCAAAACACG GATCCCGGTA TTTAGATGTC TTCTTACTCG GCTTCTTCGA    3960

GATGGAACGA ATCAAAGACA AATACGGGAG TGTGAATGAT CTCGACAGTG ACCCGAGTTA    4020

CAAAGTTTGT GGCTCTGGAT CATTACCAAT CGGATTGGCT AAGTACACTG GAATGACCA     4080

GGAATTGTTA CAAGCCGCAA CCAAACTGGA TATAGAAGTG AGAAGAACAG TCAAAGCGAA    4140

AGAGATGGTT GTTTACACGG TACAAAATAT AAAACCAGAA CTGTACCCAT GGTCCAATAG    4200

ACTAAGAAAA GGAATGCTGT TCGATGCCAA CAAAGTTGCT CTTGCTCCTC AATGTCTTCC    4260

ACTAGATAGG AGCATAAAAT TTAGAGTAAT CTTCGTGAAT GTACGGCAA TTGGATCAAT     4320

AACCTTGTTC AAAATTCCTA AGTCAATGGC ATCACTATCT CTACCCAACA CAATATCAAT    4380

CAATCTGCAG GTACACATAA AAACAGGGGT TCAGACTGAT TCTAAAGGGA TAGTTCAAAT    4440

TTTGGATGAG AAAGGCGAAA AATCACTGAA TTTCATGGTC CATCTCGGAT TGATCAAAAG    4500

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TCTAAATCCG CATCTCAATC TAGTTATCTG GGCTTCATCA GTAGAGATTA CAAGAGTGGA    4740

TGCAATTTTC CAACCTTCTT TACCTGGCGA GTTCAGATAC TATCCTAATA TTATTGCAAA    4800

AGGAGTTGGG AAAATCAAAC AATGGAACTA GTAATCTCTA TTTTAGTCCG GACGTATCTA    4860

TTAAGCCGAA GCAAATAAAG GATAATCAAA AACTTAGGAC AAAAGAGGTC AATACCAACA    4920

ACTATTAGCA GTCACACTCG CAAGAATAAG AGAGAAGGGA CCAAAAAAGT CAAATAGGAG    4980

AAATCAAAAC AAAAGGTACA GAACACCAGA ACAACAAAAT CAAAACATCC AACTCACTCA    5040

AAACAAAAAT TCCAAAGAG ACCGGCAACA CAACAAGCAC TGAACACAAT GCCAACTTCA     5100

ATACTGCTAA TTATTACAAC CATGATCATG GCATCTTTCT GCCAAATAGA TATCACAAAA    5160

CTACAGCACG TAGGTGTATT GGTCAACAGT CCCAAAGGGA TGAAGATATC ACAAAACTTT    5220

GAAACAAGAT ATCTAATTTT GAGCCTCATA CCAAAAATAG AAGACTCTAA CTCTTGTGGT    5280

GACCAACAGA TCAAGCAATA CAAGAAGTTA TTGGATAGAC TGATCATCCC TTTATATGAT    5340

GGATTAAGAT TACAGAAAGA TGTGATAGTA ACCAATCAAG AATCCAATGA AAACACTGAT    5400

CCCAGAACAA AACGATTCTT TGGAGGGGTA ATTGGAACCA TTGCTCTGGG AGTAGCAACC    5460
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TCAGCACAAA TTACAGCGGC AGTTGCTCTG GTTGAAGCCA AGCAGGCAAG ATCAGACATC    5520

GAAAAACTCA AAGAAGCAAT TAGGGACACA AACAAAGCAG TGCAGTCAGT TCAGAGCTCC    5580

ATAGGAAATT TAATAGTAGC AATTAAATCA GTCCAGGATT ATGTTAACAA AGAAATCGTG    5640

CCATCGATTG CGAGGCTAGG TTGTGAAGCA GCAGGACTTC AATTAGGAAT TGCATTAACA    5700

CAGCATTACT CAGAATTAAC AAACATATTT GGTGATAACA TAGGATCGTT ACAAGAAAAA    5760

GGAATAAAAT TACAAGGTAT AGCATCATTA TACCGCACAA ATATCACAGA AATATTCACA    5820

ACATCAACAG TTGATAAATA TGATATCTAT GATCTGTTAT TTACAGAATC AATAAAGGTG    5880

AGAGTTATAG ATGTTGACTT GAATGATTAC TCAATCACCC TCCAAGTCAG ACTCCCTTTA    5940

TTAACTAGGC TGCTGAACAC TCAGATCTAC AAAGTAGATT CCATATCATA TAACATCCAA    6000

AACAGAGAAT GGTATATCCC TCTTCCCAGC CATATCATGA CGAAAGGGGC ATTTCTAGGT    6060

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CCTGATCAAG GAGTAAAAAT TATAACACAT AAAGAATGTA GTACAATAGG TATCAACGGA    6360

ATGCTGTTCA ATACAAATAA AGAAGGAACT CTTGCATTCT ATACACCAAA TGATATAACA    6420

CTAAACAATT CTGTTGCACT TGATCCAATT GACATATCAA TCGAGCTCAA CAAGGCCAAA    6480

TCAGATCTAG AAGAATCAAA AGAATGGATA AGAAGGTCAA ATCAAAAACT AGATTCTATT    6540

GGAAATTGGC ATCAATCTAG CACTACAATC ATAATTATTT TGATAATGAT CATTATATTG    6600

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AATCGAGTGG ATCAAAATGA CAAGCCATAT GTACTAACAA ACAAATAACA TATCTACAGA    6720

TCATTAGATA TTAAAATTAT AAAAAACTTA GGAGTAAAGT TACGCAATCC AACTCTACTC    6780

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ACCAATCACG GAAAGGATGC TGGTAATGAG CTGGAGACGT CTATGGCTAC TCATGGCAAC    6900

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GTCTTCATCA TAGTGCTAAT TAATTCCATC AAAAGTGAAA AGGCCCACGA ATCATTGCTG    7020

CAAGACATAA ATAATGAGTT TATGGAAATT ACAGAAAAGA TCCAAATGGC ATCGGATAAT    7080

ACCAATGATC TAATACAGTC AGGAGTGAAT ACAAGGCTTC TTACAATTCA GAGTCATGTC    7140

CAGAATTACA TACCAATATC ATTGACACAA CAGATGTCAG ATCTTAGGAA ATTCATTAGT    7200

GAAATTACAA TTAGAAATGA TAATCAAGAA GTGCTGCCAC AAAGAATAAC ACATGATGTA    7260

GGTATAAAAC CTTTAAATCC AGATGATTTT TGGAGATGCA CGTCTGGTCT TCCATCTTTA    7320

ATGAAAACTC CAAAAATAAG GTTAATGCCA GGGCCGGGAT TATTAGCTAT GCCAACGACT    7380

GTTGATGGCT GTGTTAGAAC TCCGTCTTTA GTTATAAATG ATCTGATTTA TGCTTATACC    7440

TCAAATCTAA TTACTCGAGG TTGTCAGGAT ATAGGAAAAT CATATCAAGT CTTACAGATA    7500

GGGATAATAA CTGTAAACTC AGACTTGGTA CCTGACTTAA ATCCTAGGAT CTCTCATACC    7560
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144

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TTTAACATAA ATGACAATAG GAAGTCATGT TCTCTAGCAC TCCTAAATAT AGATGTATAT    7620

CAACTGTGTT CAACTCCCAA AGTTGATGAA AGATCAGATT ATGCATCATC AGGCATAGAA    7680

GATATTGTAC TTGATATTGT CAATTATGAT GGTTCAATCT CAACAACAAG ATTTAAGAAT    7740

AATAACATAA GCTTTGATCA ACCATATGCT GCACTATACC CATCTGTTGG ACCAGGGATA    7800

TACTACAAAG GCAAAATAAT ATTTCTCGGG TATGGAGGTC TTGAACATCC AATAAATGAG    7860

AATGTAATCT GCAACACAAC TGGGTGCCCC GGGAAAACAC AGAGAGACTG TAATCAAGCA    7920

TCTCATAGTA CTTGGTTTTC AGATAGGAGG ATGGTCAACT CCATCATTGT TGTTGACAAA    7980

GGCTTAAACT CAATTCCAAA ATTGAAAGTA TGGACGATAT CTATGCGACA AAATTACTGG    8040

GGGTCAGAAG GAAGGTTACT TCTACTAGGT AACAAGATCT ATATATATAC AAGATCTACA    8100

AGTTGGCATA GCAAGTTACA ATTAGGAATA ATTGATATTA CTGATTACAG TGATATAAGG    8160

ATAAAATGGA CATGGCATAA TGTGCTATCA AGACCAGGAA ACAATGAATG TCCATGGGGA    8220

CATTCATGTC CAGATGGATG TATAACAGGA GTATATACTG ATGCATATCC ACTCAATCCC    8280

ACAGGGAGCA TTGTGTCATC TGTCATATTA GACTCACAAA AATCGAGAGT GAACCCAGTC    8340

ATAACTTACT CAACAGCAAC CGAAAGAGTA AACGAGCTGG CCATCCTAAA CAGAACACTC    8400

TCAGCTGGAT ATACAACAAC AAGCTGCATT ACACACTATA ACAAAGGATA TTGTTTTCAT    8460

ATAGTAGAAA TAAATCATAA AAGCTTAAAC ACATTTCAAC CCATGTTGTT CAAAACAGAG    8520

ATTCCAAAAA GCTGCAGTTA ATCATAATTA ACCATAATAT GCATCAATCT ATCTATAATA    8580

CAAGTATATG ATAAGTAATC AGCAATCAGA CAATAGACAA AAGGGAAATA TAAAAAACTT    8640

AGGAGCAAAG CGTGCTCGGG AAATGGACAC TGAATCTAAC AATGGCACTG TATCTGACAT    8700

ACTCTATCCT GAGTGTCACC TTAACTCTCC TATCGTTAAA GGTAAAATAG CACAATTACA    8760

CACTATTATG AGTCTACCTC AGCCTTATGA TATGGATGAC GACTCAATAC TAGTTATCAC    8820

TAGACAGAAA ATAAAACTTA ATAAATTGGA TAAAAGACAA CGATCTATTA GAAGATTAAA    8880

ATTAATATTA ACTGAAAAAG TGAATGACTT AGGAAAATAC ACATTTATCA GATATCCAGA    8940

AATGTCAAAA GAAATGTTCA AATTATATAT ACCTGGTATT AACAGTAAAG TGACTGAATT    9000

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TAATGTGCTA TCAAAATTAG CCTCAAAAAA TGATGGAAGC AATTATGATC TTAATGAAGA    9120

AATTAATAAT ATATCGAAAG TTCACACAAC CTATAAATCA GATAAATGGT ATAATCCATT    9180

CAAAACATGG TTTACTATCA AGTATGATAT GAGAAGATTA CAAAAAGCTC GAAATGAGAT    9240

CACTTTTAAT GTTGGGAAGG ATTATAACTT GTTAGAAGAC CAGAAGAATT TCTTATTGAT    9300

ACATCCAGAA TTGGTTTTGA TATTAGATAA ACAAAACTAT AATGGTTATC TAATTACTCC    9360

TGAATTAGTA TTGATGTATT GTGACGTAGT CGAAGGCCGA TGGAATATAA GTGCATGTGC    9420

TAAGTTAGAT CCAAAATTAC AATCTATGTA TCAGAAAGGT AATAACCTGT GGGAAGTGAT    9480

AGATAAATTG TTTCCAATTA TGGGAGAAAA GACATTTGAT GTGATATCGT TATTAGAACC    9540

ACTTGCATTA TCCTTAATTC AAACTCATGA TCCTGTTAAA CAACTAAGAG GAGCTTTTTT    9600

AAATCATGTG TTATCCGAGA TGGAATTAAT ATTTGAATCT AGAGAATCGA TTAAGGAATT    9660
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TCTGAGTGTA GATTACATTG ATAAAATTTT AGATATATTT AATAAGTCTA CAATAGATGA   9720

AATAGCAGAG ATTTTCTCTT TTTTTAGAAC ATTTGGGCAT CCTCCATTAG AAGCTAGTAT   9780

TGCAGCAGAA AAGGTTAGAA AATATATGTA TATTGGAAAA CAATTAAAAT TTGACACTAT   9840

TAATAAATGT CATGCTATCT TCTGTACAAT AATAATTAAC GGATATAGAG AGAGGCATGG   9900

TGGACAGTGG CCTCCTGTGA CATTACCTGA TCATGCACAC GAATTCATCA TAAATGCTTA   9960

CGGTTCAAAC TCTGCGATAT CATATGAAAA TGCTGTTGAT TATTACCAGA GCTTTATAGG   10020

AATAAAATTC AATAAATTCA TAGAGCCTCA GTTAGATGAG GATTTGACAA TTTATATGAA   10080

AGATAAAGCA TTATCTCCAA AAAAATCAAA TTGGGACACA GTTTATCCTG CATCTAATTT   10140

ACTGTACCGT ACTAACGCAT CCAACGAATC ACGAAGATTA GTTGAAGTAT TTATAGCAGA   10200

TAGTAAATTT GATCCTCATC AGATATTGGA TTATGTAGAA TCTGGGGACT GGTTAGATGA   10260

TCCAGAATTT AATATTTCTT ATAGTCTTAA AGAAAAAGAG ATCAAACAGG AAGGTAGACT   10320

CTTTGCAAAA ATGACATACA AAATGAGAGC TACACAAGTT TTATCAGAGA CCCTACTTGC   10380

AAATAACATA GGAAAATTCT TCAAGAAAA TGGGATGGTG AAGGGAGAGA TTGAATTACT   10440

TAAGAGATTA ACAACCATAT CAATATCAGG AGTTCCACGG TATAATGAAG TGTACAATAA   10500

TTCTAAAAGC CATACAGATG ACCTTAAAAC CTACAATAAA ATAAGTAATC TTAATTTGTC   10560

TTCTAATCAG AAATCAAAGA AATTTGAATT CAAGTCAACG GATATCTACA ATGATGGATA   10620

CGAGACTGTG AGCTGTTTCC TAACAACAGA TCTCAAAAAA TACTGTCTTA ATTGGAGATA   10680

TGAATCAACA GCTCTATTTG GAGAAACTTG CAACCAAATA TTTGGATTAA ATAAAATTGTT   10740

TAATTGGTTA CACCCTCGTC TTGAAGGAAG TACAATCTAT GTAGGTGATC CTTACTGTCC   10800

TCCATCAGAT AAAGAACATA TATCATTAGA GGATCACCCT GATTCTGGTT TTTACGTTCA   10860

TAACCCAAGA GGGGGTATAG AAGGATTTTG TCAAAAATTA TGGACACTCA TATCTATAAG   10920

TGCAATACAT CTAGCAGCTG TTAGAATAGG CGTGAGGGTG ACTGCAATGG TTCAAGGAGA   10980

CAATCAAGCT ATAGCTGTAA CCACAAGAGT ACCCAACAAT TATGACTACA GAGTTAAGAA   11040

GGAGATAGTT TATAAAGATG TAGTGAGATT TTTTGATTCA TTAAGAGAAG TGATGGATGA   11100

TCTAGGTCAT GAACTTAAAT TAAATGAAAC GATTATAAGT AGCAAGATGT CATATATATAG   11160

CAAAAGAATC TATTATGATG GGAGAATTCT TCCTCAAGCT CTAAAAGCAT TATCTAGATG   11220

TGTCTTCTGG TCAGAGACAG TAATAGACGA AACAAGATCA GCATCTTCAA ATTTGGCAAC   11280

ATCATTTGCA AAAGCAATTG AGAATGGTTA TTCACCTGTT CTAGGATATG CATGCTCAAT   11340

TTTTAAGAAT ATTCAACAAC TATATATTGC CCTTGGGATG AATATCAATC CAACTATAAC   11400

ACAGAATATC AGAGATCAGT ATTTTAGGAA TCCAAATTGG ATGCAATATG CCTCTTTAAT   11460

ACCTGCTAGT GTTGGGGGAT TCAATTACAT GGCCATGTCA AGATGTTTTG TAAGGAATAT   11520

TGGTGATCCA TCAGTTGCCG CATTGGCTGA TATTAAAAGA TTTATTAAGG CGAATCTATT   11580

AGACCGAAGT GTTCTTTATA GGATTATGAA TCAAGAACCA GGTGAGTCAT CTTTTTTGGA   11640

CTGGGCTTCA GATCCATATT CATGCAATTT ACCACAATCT CAAAATATAA CCACCATGAT   11700

AAAAAAATATA ACAGCAAGGA ATGTATTACA AGATTCACCA AATCCATTAT TATCTGGATT   11760
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ATTCACAAAT ACAATGATAG AAGAAGATGA AGAATTAGCT GAGTTCCTGA TGGACAGGAA    11820

GGTAATTCTC CCTAGAGTTG CACATGATAT TCTAGATAAT TCTCTCACAG GAATTAGAAA    11880

TGCCATAGCT GGAATGTTAG ATACGACAAA ATCACTAATT CGGGTTGGCA TAAATAGAGG    11940

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GGGTCTTGAG GGGTTTTTTG CTGAAAGGAG GAACTATATA CGCGTCGACG GGCCCCGCGC    15660
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(2) INFORMATION FOR SEQ ID NO:15:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 15666 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

148

(ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

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CTTTAAATTA ACTTAGGATT AAAGACATTG ACTAGAAGGT CAAGAAAAGG GAACTCTATA   120

ATTTCAAAAA TGTTGAGCCT ATTTGATACA TTTAATGCAC GTAGGCAAGA AAACATAACA   180

AAATCAGCCG GTGGAGCTAT CATTCCTGGA CAGAAAAATA CTGTCTCTAT ATTCGCCCTT   240

GGACCGACAA TAACTGATGA TAATGAGAAA ATGACATTAG CTCTTCTATT TCTATCTCAT   300

TCACTAGATA ATGAGAAACA ACATGCACAA AGGGCAGGGT CTTGGTGTC TTTATTGTCA   360

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GATAGTAAAT TTGATCCTCA TCAGATATTG GATTATGTAG AATCTGGGGA CTGGTTAGAT   10260

GATCCAGAAT TTAATATTTC TTATAGTCTT AAAGAAAAAG AGATCAAACA GGAAGGTAGA   10320
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CTCTTTGCAA AAATGACATA CAAAATGAGA GCTACACAAG TTTTATCAGA GACCCTACTT    10380

GCAAATAACA TAGGAAAATT CTTTCAAGAA AATGGGATGG TGAAGGGAGA GATTGAATTA    10440

CTTAAGAGAT TAACAACCAT ATCAATATCA GGAGTTCCAC GGTATAATGA AGTGTACAAT    10500

AATTCTAAAA GCCATACAGA TGACCTTAAA ACCTACAATA AAATAAGTAA TCTTAATTTG    10560

TCTTCTAATC AGAAATCAAA GAAATTTGAA TTCAAGTCAA CGGATATCTA CAATGATGGA    10620

TACGAGACTG TGAGCTGTTT CCTAACAACA GATCTCAAAA AATACTGTCT TAATTGGAGA    10680

TATGAATCAA CAGCTCTATT TGGAGAAACT TGCAACCAAA TATTTGGATT AAATAAATTG    10740

TTTAATTGGT TACACCCTCG TCTTGAAGGA AGTACAATCT ATGTAGGTGA TCCTTACTGT    10800

CCTCCATCAG ATAAAGAACA TATATCATTA GAGGATCACC CTGATTCTGG TTTTTACGTT    10860

CATAACCCAA GAGGGGGTAT AGAAGGATTT TGTCAAAAAT TATGGACACT CATATCTATA    10920

AGTGCAATAC ATCTAGCAGC TGTTAGAATA GGCGTGAGGG TGACTGCAAT GGTTCAAGGA    10980

GACAATCAAG CTATAGCTGT AACCACAAGA GTACCCAACA ATTATGACTA CAGAGTTAAG    11040

AAGGAGATAG TTTATAAAGA TGTAGTGAGA TTTTTTGATT CATTAAGAGA AGTGATGGAT    11100

GATCTAGGTC ATGAACTTAA ATTAAATGAA ACGATTATAA GTAGCAAGAT GTTCATATAT    11160

AGCAAAAGAA TCTATTATGA TGGGAGAATT CTTCCTCAAG CTCTAAAAGC ATTATCTAGA    11220

TGTGTCTTCT GGTCAGAGAC AGTAATAGAC GAAACAAGAT CAGCATCTTC AAATTTGGCA    11280

ACATCATTTG CAAAAGCAAT TGAGAATGGT TATTCACCTG TTCTAGGATA TGCATGCTCA    11340

ATTTTTAAGA ATATTCAACA ACTATATATT GCCCTTGGGA TGAATATCAA TCCAACTATA    11400

ACACAGAATA TCAGAGATCA GTATTTTAGG AATCCAAATT GGATGCAATA TGCCTCTTTA    11460

ATACCTGCTA GTGTTGGGGG ATTCAATTAC ATGGCCATGT CAAGATGTTT TGTAAGGAAT    11520

ATTGGTGATC CATCAGTTGC CGCATTGGCT GATATTAAAA GATTTATTAA GGCGAATCTA    11580

TTAGACCGAA GTGTTCTTTA TAGGATTATG AATCAAGAAC CAGGTGAGTC ATCTTTTTTG    11640

GACTGGGCTT CAGATCCATA TTCATGCAAT TTACCACAAT CTCAAAATAT AACCACCATG    11700

ATAAAAAATA TAACAGCAAG GAATGTATTA CAAGATTCAC CAAATCCATT ATTATCTGGA    11760

TTATTCACAA ATACAATGAT AGAAGAAGAT GAAGAATTAG CTGAGTTCCT GATGGACAGG    11820

AAGGTAATTC TCCCTAGAGT TGCACATGAT ATTCTAGATA ATTCTCTCAC AGGAATTAGA    11880

AATGCCATAG CTGGAATGTT AGATACGACA AAATCACTAA TTCGGGTTGG CATAAATAGA    11940

GGAGGACTGA CATATAGTTT GTTGAGGAAA ATCAGTAATT ACGATCTAGT ACAATATGAA    12000

ACACTAAGTA GGACTTTGCG ACTAATTGTA AGTGATAAAA TCAAGTATGA AGATATGTGT    12060

TCGGTAGACC TTGCCATAGC ATTGCGACAA AAGATGTGGA TTCATTTATC AGGAGGAAGG    12120

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GGATCAGAAC ATTGTAAAAT ATGTTATTCT TCAGATGGCA CAAACCCATA TACTTGGATG    12240

TATTTACCCG GTAATATCAA AATAGGATCA GCAGAAACAG GTATATCGTC ATTAAGAGTT    12300

CCTTATTTTG GATCAGTCAC TGATGAAAGA TCTGAAGCAC AATTAGGATA TATCAAGAAT    12360

CTTAGTAAAC CTGCAAAAGC CGCAATAAGA ATAGCAATGA TATATACATG GGCATTTGGT    12420
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AATGATGAGA TATCTTGGAT GGAAGCCTCA CAGATAGCAC AAACACGTGC AAATTTTACA    12480

CTAGATAGTC TCAAAATTTT AACACCGGTA GCTACATCAA CAAATTTATC ACACAGATTA    12540

AAGGATACTG CAACTCAGAT GAAATTCTCC AGTACATCAT TGATCAGAGT CAGCAGATTC    12600

ATAACAATGT CCAATGATAA CATGTCTATC AAAGAAGCTA ATGAAACCAA AGATACTAAT    12660

CTTATTTATC AACAAATAAT GTTAACAGGA TTAAGTGTTT CGAATATTT  ATTTAGATTA    12720

AAAGAAACCA CAGGACACAA CCCTATAGTT ATGCATCTGC ACATAGAAGA TGAGTGTTGT    12780

ATTAAAGAAA GTTTTAATGA TGAACATATT AATCCAGAGT CTACATTAGA ATTAATTCGA    12840

TATCCTGAAA GTAATGAATT TATTTATGAT AAAGACCCAC TCAAAGATGT GGACTTATCA    12900

AAACTTATGG TTATTAAAGA CCATTCTTAC ACAATTGATA TGAATTATTG GGATGATACT    12960

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TTAGATCGAG ATAATTTAAA AGAGATAATA GTTATTGCAA ATGATGATGA TATTAATAGC    13080

TTAATCACTG AATTTTTGAC TCTTGACATA CTTGTATTTC TCAAGACATT TGGTGGATTA    13140

TTAGTAAATC AATTTGCATA CACTCTTTAT AGTCTAAAAA TAGAAGGTAG GGATCTCATT    13200

TGGGATTATA TAATGAGAAC ACTGAGAGAT ACTTCCCATT CAATATTAAA AGTATTATCT    13260

AATGCATTAT CTCATCCTAA AGTATTCAAG AGGTTCTGGG ATTGTGGAGT TTTAAACCCT    13320

ATTTATGGTC CTAATACTGC TAGTCAAGAC CAGATAAAAC TTGCCCTATC TATATGTGAA    13380

TATTCACTAG ATCTATTTAT GAGAGAATGG TTGAATGGTG TATCACTTGA AATATACATT    13440

TGTGACAGCG ATATGGAAGT TGCAAATGAT AGGAAACAAG CCTTTATTTC TAGACACCTT    13500

TCATTTGTTT GTTGTTTAGC AGAAATTGCA TCTTTCGGAC CTAACCTGTT AAACTTAACA    13560

TACTTGGAGA GACTTGATCT ATTGAAACAA TATCTTGAAT TAAATATTAA AGAAGACCCT    13620

ACTCTTAAAT ATGTACAAAT ATCTGGATTA TTAATTAAAT CGTTCCCATC AACTGTAACA    13680

TACGTAAGAA AGACTGCAAT CAAATATCTA AGGATTCGCG GTATTAGTCC ACCTGAGGTA    13740

ATTGATGATT GGGATCCGGT AGAAGATGAA AATATGCTGG ATAACATTGT CAAAACTATA    13800

AATGATAACT GTAATAAAGA TAATAAAGGG AATAAAATTA ACAATTTCTG GGGACTAGCA    13860

CTTAAGAACT ATCAAGTCCT TAAAATCAGA TCTATAACAA GTGATTCTGA TGATAATGAT    13920

AGACTAGATG CTAATACAAG TGGTTTGACA CTTCCTCAAG GAGGGAATTA TCTATCGCAT    13980

CAATTGAGAT TATTCGGAAT CAACAGCACT AGTTGTCTGA AAGCTCTTGA GTTATCACAA    14040

ATTTTAATGA AGGAAGTCAA TAAAGACAAG GACAGGCTCT TCCTGGGAGA AGGAGCAGGA    14100

GCTATGCTAG CATGTTATGA TGCCACATTA GGACCTGCAG TTAATTATTA TAATTCAGGT    14160

TTGAATATAA CAGATGTAAT TGGTCAACGA GAATTGAAAA TATTTCCTTC AGAGGTATCA    14220

TTAGTAGGTA AAAAATTAGG AAATGTGACA CAGATTCTTA ACAGGGTAAA AGTACTGTTC    14280

AATGGGAATC CTAATTCAAC ATGGATAGGA AATATGGAAT GTGAGAGCTT AATATGGAGT    14340

GAATTAAATG ATAAGTCCAT TGGATTAGTA CATTGTGATA TGGAAGGAGC TATCGGTAAA    14400

TCAGAAGAAA CTGTTCTACA TGAACATTAT AGTGTTATAA GAATTACATA CTTGATTGGG    14460

GATGATGATG TTGTTTTAGT TTCCAAAATT ATACCTACAA TCACTCCGAA TTGGTCTAGA    14520
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ATACTTTATC TATATAAATT ATATTGGAAA GATGTAAGTA TAATATCACT CAAAACTTCT   14580

AATCCTGCAT CAACAGAATT ATATCTAATT TCGAAAGATG CATATTGTAC TATAATGGAA   14640

CCTAGTGAAA TTGTTTTATC AAAACTTAAA AGATTGTCAC TCTTGGAAGA AAATAATCTA   14700

TTAAAATGGA TCATTTTATC AAAGAAGAGG AATAATGAAT GGTTACATCA TGAAATCAAA   14760

GAAGGAGAAA GAGATTATGG AATCATGAGA CCATATCATA TGGCACTACA AATCTTTGGA   14820

TTTCAAATCA ATTTAAATCA TCTGGCGAAA GAATTTTTAT CAACCCCAGA TCTGACTAAT   14880

ATCAACAATA TAATCCAAAG TTTTCAGCGA ACAATAAAGG ATGTTTTATT TGAATGGATT   14940

AATATAACTC ATGATGATAA GAGACATAAA TTAGGCGGAA GATATAACAT ATTCCCACTG   15000

AAAAATAAGG GAAAGTTAAG ACTGCTATCG AGAAGACTAG TATTAAGTTG GATTTCATTA   15060

TCATTATCGA CTCGATTACT TACAGGTCGC TTTCCTGATG AAAAATTTGA ACATAGAGCA   15120

CAGACTGGAT ATGTATCATT AGCTGATACT GATTTAGAAT CATTAAAGTT ATTGTCGAAA   15180

AACATCATTA AGAATTACAG AGAGTGTATA GGATCAATAT CATATTGGTT TCTAACCAAA   15240

GAAGTTAAAA TACTTATGAA ATTGATCGGT GGTGCTAAAT TATTAGGAAT TCCCAGACAA   15300

TATAAAGAAC CCGAAGACCA GTTATTAGAA AACTACAATC AACATGATGA ATTTGATATC   15360

GATTAAAACA TAAATACAAT GAAGATATAT CCTAACCTTT ATCTTTAAGC CTAGGAATAG   15420

ACAAAAAGTA AGAAAAACAT GTAATATATA TATACCAAAC AGAGTTCTTC TCTTGTTTGG   15480

TGGGTCGGCA TGGCATCTCC ACCTCCTCGC GGTCCGGACC TGGGCATCCG AAGGAGGACG   15540

CACGTCCACT CGGATGGCTA AGGGAGAGCC TGCAGTAGCA TAACCCCTTG GGGCCTCTAA   15600

ACGGGTCTTG AGGGGTTTTT TGCTGAAAGG AGGAACTATA TACGCGTCGA CGGGCCCCGC   15660

GCTCAC                                                             15666
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(2) INFORMATION FOR SEQ ID NO:16:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 28 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

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GGATTTGCGC GCAATTTAAA TCATCTGG                                           28
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(2) INFORMATION FOR SEQ ID NO:17:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 70 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CCCAGGTCGG ACCGCGAGGA GGTGGAGATG CCATGCCAGC CCACCAAAAC AAGAGAAGAA   60

CTCTGTTTGG   70

(2) INFORMATION FOR SEQ ID NO:18:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 45 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GGCCCGTCGA CGCGTAATAC GACTCACTAT AGGACCAAAC AAGAG   45

(2) INFORMATION FOR SEQ ID NO:19:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 16 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CGGCATCACG TGCTAC   16

(2) INFORMATION FOR SEQ ID NO:20:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 6843 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

ATGTTGAGCC TATTTGATAC ATTTAATGCA CGTAGGCAAG AAAACATAAC AAAATCAGCC   60

GGTGGAGCTA TCATTCCTGG ACAGAAAAAT ACTGTCTCTA TATTCGCCCT TGGACCGACA   120

ATAACTGATG ATAATGAGAA AATGACATTA GCTCTTCTAT TTCTATCTCA TTCACTAGAT   180

AATGAGAAAC AACATGCACA AAGGGCAGGG TTCTTGGTGT CTTTATTGTC AATGGCTTAT   240

GCCAATCCAG AGCTCTACCT AACAACAAAT GGAAGTAATG CAGATGTCAA GTATGTCATA   300

TACATGATTG AGAAAGATCT AAAACGGCAA AAGTATGGAG GATTTGTGGT TAAGACGAGA   360

GAGATGATAT ATGAAAAGAC AACTGATTGG ATATTTGGAA GTGACCTGGA TTATGATCAG   420

GAAACTATGT TGCAGAACGG CAGGAACAAT TCAACAATTG AAGACCTTGT CCACACATTT   480

GGGTATCCAT CATGTTTAGG AGCTCTTATA ATACAGATCT GGATAGTTCT GGTCAAAGCT   540

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ATCACTAGTA TCTCAGGGTT AAGAAAAGGC TTTTTCACCC GATTGGAAGC TTTCAGACAA    600

GATGGAACAG TGCAGGCAGG GCTGGTATTG AGCGGTGACA CAGTGGATCA GATTGGGTCA    660

ATCATGCGGT CTCAACAGAG CTTGGTAACT CTTATGGTTG AAACATTAAT AACAATGAAT    720

ACCAGCAGAA ATGACCTCAC AACCATAGAA AAGAATATAC AAATTGTTGG CAACTACATA    780

AGAGATGCAG GTCTCGCTTC ATTCTTCAAT ACAATCAGAT ATGGAATTGA GACCAGAATG    840

GCAGCTTTGA CTCTATCCAC TCTCAGACCA GATATCAATA GATTAAAAGC TTTGATGGAA    900

CTGTATTTAT CAAAGGGACC ACGCGCTCCT TTCATCTGTA TCCTCAGAGA TCCTATACAT    960

GGTGAGTTCG CACCAGGCAA CTATCCTGCC ATATGGAGCT ATGCAATGGG GGTGGCAGTT   1020

GTACAAAATA GAGCCATGCA ACAGTATGTG ACGGGAAGAT CATATCTAGA CATTGATATG   1080

TTCCAGCTAG GACAAGCAGT AGCACGTGAT GCCGAAGCTC AAATGAGCTC AACACTGGAA   1140

GATGAACTTG GAGTGACACA CGAATCTAAA GAAAGCTTGA AGAGACATAT AAGGAACATA   1200

AACAGTTCAG AGACATCTTT CCACAAACCG ACAGGTGGAT CAGCCATAGA GATGGCAATA   1260

GATGAAGAGC CAGAACAATT CGAACATAGA GCAGATCAAG AACAAAATGG AGAACCTCAA   1320

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GCTACAGAAT CTGACAATAT CAAGACCGAA CAACAAAACA TCAGAGACAG ACTAAACAAG   1440

AGACTCAACG ACAAGAAGAA ACAAAGCAGT CAACCACCCA CTAATCCCAC AAACAGAACA   1500

AACCAGGACG AAATAGATGA TCTGTTTAAC GCATTGGAA GCAACTAAGT CGACGATCCG   1560

GCTGCTAACA AAGCCCGAAA GGAAGCTGAG TTGGCTGCTG CCACCGCTGA GCAATAACTA   1620

GCATAACCCC TTGGGGCCTC TAAACGGGTC TTGAGGGGTT TTTTGCTGAA AGGAGGAACT   1680

ATATCCGGAT CGAGATCAAT TCTGTGAGCG TATGGCAAAC GAAGGAAAAA TAGTTATAGT   1740

AGCCGCACTC GATGGGACAT TTCAACGTAA ACCGTTTAAT AATATTTTGA ATCTTATTCC   1800

ATTATCTGAA ATGGTGGTAA AACTAACTGC TGTGTGTATG AAATGCTTTA AGGAGGCTTC   1860

CTTTTCTAAA CGATTGGGTG AGGAAACCGA GATAGAAATA ATAGGAGGTA ATGATATGTA   1920

TCAATCGGTG TGTAGAAAGT GTTACATCGA CTCATAATAT TATATTTTTT ATCTAAAAAA   1980

CTAAAAATAA ACATTGATTA AATTTTAATA TAATACTTAA AAATGGATGT TGTGTCGTTA   2040

GATAAACCGT TTATGTATTT TGAGGAAATT GATAATGAGT TAGATTACGA ACCAGAAAGT   2100

GCAAATGAGG TCGCAAAAAA ACTGCCGTAT CAAGGACAGT TAAAACTATT ACTAGGAGAA   2160

TTATTTTTTC TTAGTAAGTT ACAGCGACAC GGTATATTAG ATGGTGCCAC CGTAGTGTAT   2220

ATAGGATCTG CTCCCGGTAC ACATATACGT TATTTGAGAG ATCATTTCTA TAATTTAGGA   2280

GTGATCATCA AATGGATGCT AATTGACGGC CGCCATCATG ATCCTATTTT AAATGGATTG   2340

CGTGATGTGA CTCTAGTGAC TCGGTTCGTT GATGAGGAAT ATCTACGATC CATCAAAAAA   2400

CAACTGCATC CTTCTAAGAT TATTTTAATT TCTGATGTGA GATCCAAACG AGGAGGAAAT   2460

GAACCTAGTA CGGCGGATTT ACTAAGTAAT TACGCTCTAC AAAATGTCAT GATTAGTATT   2520

TTAAACCCCG TGGCGTCTAG TCTTAAATGG AGATGCCCGT TTCCAGATCA ATGGATCAAG   2580

GACTTTTATA TCCCACACGG TAATAAAAATG TTACAACCTT TTGCTCCTTC ATATTCAGGG   2640
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CCGTCGTTTT ACAACGTCGT GACTGGGAAA ACCCTGGCGT TACCCAACTT AATCGCCTTG   2700

CAGCACATCC CCCTTTCGCC AGCTGGCGTA ATAGCGAAGA GGCCCGCACC GATCGCCCTT   2760

CCCAACAGTT GCGCAGCCTG AATGGCGAAT GGCGCGACGC GCCCTGTAGC GGCGCATTAA   2820

GCGCGGCGGG TGTGGTGGTT ACGCGCAGCG TGACCGCTAC ACTTGCCAGC GCCCTAGCGC   2880

CCGCTCCTTT CGCTTTCTTC CCTTCCTTTC TCGCCACGTT CGCCGGCTTT CCCCGTCAAG   2940

CTCTAAATCG GGGGCTCCCT TTAGGGTTCC GATTTAGTGC TTTACGCAC CTCGACCCCA    3000

AAAAACTTGA TTAGGGTGAT GGTTCACGTA GTGGGCCATC GCCCTGATAG ACGGTTTTTC   3060

GCCCTTTGAC GTTGGAGTCC ACGTTCTTTA ATAGTGGACT CTTGTTCCAA ACTGGAACAA   3120

CACTCAACCC TATCTCGGTC TATTCTTTTG ATTTATAAGG GATTTTGCCG ATTTCGGCCT   3180

ATTGGTTAAA AAATGAGCTG ATTTAACAAA AATTTAACGC GAATTTTAAC AAAATATTAA   3240

CGTTTACAAT TTCCCAGGTG GCACTTTTCG GGGAAATGTG CGCGGAACCC CTATTTGTTT   3300

ATTTTTCTAA ATACATTCAA ATATGTATCC GCTCATGAGA CAATAACCCT GATAAATGCT   3360

TCAATAATAT TGAAAAAGGA AGAGTATGAG TATTCAACAT TTCCGTGTCG CCCTTATTCC   3420

CTTTTTTGCG GCATTTTGCC TTCCTGTTTT TGCTCACCCA GAAACGCTGG TGAAAGTAAA   3480

AGATGCTGAA GATCAGTTGG GTGCACGAGT GGGTTACATC GAACTGGATC TCAACAGCGG   3540

TAAGATCCTT GAGAGTTTTC GCCCCGAAGA ACGTTTTCCA ATGATGAGCA CTTTTAAAGT   3600

TCTGCTATGT GGCGCGGTAT TATCCCGTAT TGACGCCGGG CAAGAGCAAC TCGGTCGCCG   3660

CATACACTAT TCTCAGAATG ACTTGGTTGA GTACTCACCA GTCACAGAAA AGCATCTTAC   3720

GGATGGCATG ACAGTAAGAG AATTATGCAG TGCTGCCATA ACCATGAGTG ATAACACTGC   3780

GGCCAACTTA CTTCTGACAA CGATCGGAGG ACCGAAGGAG CTAACCGCTT TTTTGCACAA   3840

CATGGGGGAT CATGTAACTC GCCTTGATCG TTGGGAACCG GAGCTGAATG AAGCCATACC   3900

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TAAAGTTGCA GGACCACTTC TGCGCTCGGC CCTTCCGGCT GGCTGGTTTA TTGCTGATAA   4080

ATCTGGAGCC GGTGAGCGTG GGTCTCGCGG TATCATTGCA GCACTGGGGC CAGATGGTAA   4140

GCCCTCCCGT ATCGTAGTTA TCTACACGAC GGGGAGTCAG GCAACTATGG ATGAACGAAA   4200

TAGACAGATC GCTGAGATAG GTGCCTCACT GATTAAGCAT TGGTAACTGT CAGACCAAGT   4260

TTACTCATAT ATACTTTAGA TTGATTTAAA ACTTCATTTT TAATTTAAAA GGATCTAGGT   4320

GAAGATCCTT TTTGATAATC TCATGACCAA AATCCCTTAA CGTGAGTTTT CGTTCCACTG   4380

AGCGTCAGAC CCCGTAGAAA AGATCAAAGG ATCTTCTTGA GATCCTTTTT TTCTGCGCGT   4440

AATCTGCTGC TTGCAAACAA AAAAACCACC GCTACCAGCG GTGGTTTGTT TGCCGGATCA   4500

AGAGCTACCA ACTCTTTTTC CGAAGGTAAC TGGCTTCAGC AGAGCGCAGA TACCAAATAC   4560

TGTCCTTCTA GTGTAGCCGT AGTTAGGCCA CCACTTCAAG AACTCTGTAG CACCGCCTAC   4620

ATACCTCGCT CTGCTAATCC TGTTACCAGT GGCTGCTGCC AGTGGCGATA AGTCGTGTCT   4680

TACCGGGTTG GACTCAAGAC GATAGTTACC GGATAAGGCG CAGCGGTCGG GCTGAACGGG   4740
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GGGTTCGTGC ACACAGCCCA GCTTGGAGCG AACGACCTAC ACCGAACTGA GATACCTACA    4800

GCGTGAGCTA TGAGAAAGCG CCACGCTTCC CGAAGGGAGA AAGGCGGACA GGTATCCGGT    4860

AAGCGGCAGG GTCGGAACAG GAGAGCGCAC GAGGGAGCTT CCAGGGGGAA ACGCCTGGTA    4920

TCTTTATAGT CCTGTCGGGT TTCGCCACCT CTGACTTGAG CGTCGATTTT TGTGATGCTC    4980

GTCAGGGGGG CGGAGCCTAT GGAAAAACGC CAGCAACGCG GCCTTTTTAC GGTTCCTGGC    5040

CTTTTGCTGG CCTTTTGCTC ACATGTTCTT TCCTGCGTTA TCCCCTGATT CTGTGGATAA    5100

CCGTATTACC GCCTTTGAGT GAGCTGATAC CGCTCGCCGC AGCCGAACGA CCGAGCGCAG    5160

CGAGTCAGTG AGCGAGGAAG CGGAAGAGCG CCCAATACGC AAACCGCCTC TCCCCGCGCG    5220

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GCGCAACGCA ATTAATGTGA GTTAGCTCAC TCATTAGGCA CCCCAGGCTT TACACTTTAT    5340

GCTTCCGGCT CGTATGTTGT GTGGAATTGT GAGCGGATAA CAATTTCACA CAGGAAACAG    5400

CTATGACCAT GATTACGCCA AGCTTTTGCG ATCAATAAAT GGATCACAAC CAGTATCTCT    5460

TAACGATGTT CTTCGCAGAT GATGATTCAT TTTTTAAGTA TTTGGCTAGT CAAGATGATG    5520

AATCTTCATT ATCTGATATA TTGCAAATCA CTCAATATCT AGACTTTCTG TTATTATTAT    5580

TGATCCAATC AAAAAATAAA TTAGAAGCCG TGGGTCATTG TTATGAATCT CTTTCAGAGG    5640

AATACAGACA ATTGACAAAA TTCACAGACT TTCAAGATTT TAAAAAACTG TTTAACAAGG    5700

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GACTATGGAC GCATGATAAG AATAATTTTG AAGCATTGGA AGCAACTAAA CTATGTGATG    6120

TCTTGGAATC AATTACAGAT TTCTCCGTGA TAGGTATCGA TGAAGGACAG TTCTTTCCAG    6180

ACATTGTTGA ATTGATCTCG ATCCCGCGAA ATTAATACGA CTCACTATAG GGAGACCACA    6240

ACGGTTTCCC TCTAGCGGGA TCAATTCCGC CCCTCTCCCT CCCCCCCCCC TAACGTTACT    6300

GGCCGAAGCC GCTTGGAATA AGGCCGGTGT GCGTTTGTCT ATATGTTATT TTCCACCATA    6360

TTGCCGTCTT TTGGCAATGT GAGGGCCCGG AAACCTGGCC CTGTCTTCTT GACGAGCATT    6420

CCTAGGGGTC TTTCCCCTCT CGCCAAAGGA ATGCAAGGTC TGTTGAATGT CGTGAAGGAA    6480

GCAGTTCCTC TGGAAGCTTC TTGAAGACAA ACAACGTCTG TAGCGACCCT TTGCAGGCAG    6540

CGGAACCCCC CACCTGGCGA CAGGTGCCTC TGCGGCCAAA AGCCACGTGT ATAAGATACA    6600

CCTGCAAAGG CGGCACAACC CCAGTGCCAC GTTGTGAGTT GGATAGTTGT GGAAAGAGTC    6660

AAATGGCTCT CCTCAAGCGT ATTCAACAAG GGGCTGAAGG ATGCCCAGAA GGTACCCCAT    6720

TGTATGGGAT CTGATCTGGG GCCTCGGTGC ACATGCTTTA CATGTGTTTA GTCGAGGTTA    6780

AAAAACGTCT AGGCCCCCCG AACCACGGGG ACGTGGTTTT CCTTTGAAAA ACACGATAAT    6840
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ACC   6843

(2) INFORMATION FOR SEQ ID NO:21:

       (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 7107 base pairs
           (B) TYPE: nucleic acid
           (C) STRANDEDNESS: single
           (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: cDNA

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

ATGGAAAGCG ACGCTAAAAA CTATCAAATC ATGGATTCTT GGGAAGAGGA ATCAAGAGAT   60

AAATCAACTA ATATCTCCTC GGCCCTCAAC ATCATTGAAT TCATACTCAG CACCGACCCC   120

CAAGAAGACT TATCGGAAAA CGACACAATC AACACAAGAA CCCAGCAACT CAGTGCCACC   180

ATCTGTCAAC CAGAAATCAA ACCAACAGAA ACAAGTGAGA AAGATAGTGG ATCAACTGAC   240

AAAAATAGAC AGTCCGGGTC ATCACACGAA TGTACAACAG AAGCAAAAGA TAGAAATATT   300

GATCAGGAAA CTGTACAGAG AGGACCTGGG AGAAGAAGCA GCTCAGATAG TAGAGCTGAG   360

ACTGTGGTCT CTGGAGGAAT CCCCAGAAGC ATCACAGATT CTAAAAATGG AACCCAAAAC   420

ACGGAGGATA TTGATCTCAA TGAAATTAGA AAGATGGATA AGGACTCTAT TGAGGGGAAA   480

ATGCGACAAT CTGCAAATGT TCCAAGCGAG ATATCAGGAA GTGATGACAT ATTTACAACA   540

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TCAGACTACA GATCCACATC AAAAGGGCAG AAGAAAATCT CAAAGACAAC AACCACCAAC   840

ACCGACACAA AGGGGCAAAC AGAAATACAG ACAGAATCAT CAGAAACACA ATCCTCATCA   900

TGGAATCTCA TCATCGACAA CAACACCGAC CGGAACGAAC AGACAAGCAC AACTCCTCCA   960

ACAACAACTT CCAGATCAAC TTATACAAAA GAATCGATCC GAACAAACTC TGAATCCAAA   1020

CCCAAGACAC AAAAGACAAA TGGAAAGGAA AGGAAGGATA CAGAAGAGAG CAATCGATTT   1080

ACAGAGAGGG CAATTACTCT ATTGCAGAAT CTTGGTGTAA TTCAATCCAC ATCAAAACTA   1140

GATTTATATC AAGACAAACG AGTTGTATGT GTAGCAAATG TACTAAACAA TGTAGATACT   1200

GCATCAAAGA TAGATTTCCT GGCAGGATTA GTCATAGGGG TTTCAATGGA CAACGACACA   1260

AAATTAACAC AGATACAAAA TGAAATGCTA AACCTCAAAG CAGATCTAAA GAAAATGGAC   1320

GAATCACATA GAAGATTGAT AGAAAATCAA AGAGAACAAC TGTCATTGAT CACGTCACTA   1380

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GAGAGAGTAT CCATGATCAA AACAAAATTG AAAGAAGAAA AGATCAAGAA GACCAGGTTT   1500

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GGAGATACAC TAGAGAACGA TGTACAAGTT AAAATCAGAGA TATTAAGTTC ATACAATGAG   1620

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AAGGATGCCC AGAAGGTACC CCATTGTATG GGATCTGATC TGGGGCCTCG GTGCACATGC    7020

TTTACATGTG TTTAGTCGAG GTTAAAAAAC GTCTAGGCCC CCCGAACCAC GGGGACGTGG    7080

TTTTCCTTTG AAAAACACGA TAATACC                                       7107
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(2) INFORMATION FOR SEQ ID NO:22:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 12011 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

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ATGGACACTG AATCTAACAA TGGCACTGTA TCTGACATAC TCTATCCTGA GTGTCACCTT      60

AACTCTCCTA TCGTTAAAGG TAAAATAGCA CAATTACACA CTATTATGAG TCTACCTCAG     120

CCTTATGATA TGGATGACGA CTCAATACTA GTTATCACTA GACAGAAAAT AAAACTTAAT     180

AAATTGGATA AAGACAACG ATCTATTAGA AGATTAAAAT TAATATTAAC TGAAAAAGTG      240

AATGACTTAG GAAAATACAC ATTTATCAGA TATCCAGAAA TGTCAAAAGA AATGTTCAAA     300

TTATATATAC CTGGTATTAA CAGTAAAGTG ACTGAATTAT TACTTAAAGC AGATAGAACA     360
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TATAACTTGT TAGAAGACCA GAAGAATTTC TTATTGATAC ATCCAGAATT GGTTTTGATA    660

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ACTCATGATC CTGTTAAACA ACTAAGAGGA GCTTTTTTAA ATCATGTGTT ATCCGAGATG    960

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AACGAATCAC GAAGATTAGT TGAAGTATTT ATAGCAGATA GTAAATTTGA TCCTCATCAG   1560

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GGATTTTGTC AAAAATTATG GACACTCATA TCTATAAGTG CAATACATCT AGCAGCTGTT   2280

AGAATAGGCG TGAGGGTGAC TGCAATGGTT CAAGGAGACA ATCAAGCTAT AGCTGTAACC   2340

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GTGAGATTTT TTGATTCATT AAGAGAAGTG ATGGATGATC TAGGTCATGA ACTTAAATTA   2460
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CTTTATAGTC TAAAAATAGA AGGTAGGGAT CTCATTTGGG ATTATATAAT GAGAACACTG   4560
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CAGAAAGTGC AAATGAGGTC GCAAAAAAAC TGCCGTATCA AGGACAGTTA AAACTATTAC   7320

TAGGAGAATT ATTTTTTCTT AGTAAGTTAC AGCGACACGG TATATTAGAT GGTGCCACCG   7380

TAGTGTATAT AGGATCTGCT CCCGGTACAC ATATACGTTA TTTGAGAGAT CATTTCTATA   7440

ATTTAGGAGT GATCATCAAA TGGATGCTAA TTGACGGCCG CCATCATGAT CCTATTTTAA   7500

ATGGATTGCG TGATGTGACT CTAGTGACTC GGTTCGTTGA TGAGGAATAT CTACGATCCA   7560

TCAAAAAACA ACTGCATCCT TCTAAGATTA TTTTAATTTC TGATGTGAGA TCCAAACGAG   7620

GAGGAAATGA ACCTAGTACG GCGGATTTAC TAAGTAATTA CGCTCTACAA AATGTCATGA   7680

TTAGTATTTT AAACCCCGTG GCGTCTAGTC TTAAATGGAG ATGCCCGTTT CCAGATCAAT   7740

GGATCAAGGA CTTTTATATC CCACACGGTA ATAAAATGTT ACAACCTTTT GCTCCTTCAT   7800

ATTCAGGGCC GTCGTTTTAC AACGTCGTGA CTGGGAAAAC CCTGGCGTTA CCCAACTTAA   7860

TCGCCTTGCA GCACATCCCC CTTTCGCCAG CTGGCGTAAT AGCGAAGAGG CCCGCACCGA   7920

TCGCCCTTCC CAACAGTTGC GCAGCCTGAA TGGCGAATGG CGCGACGCGC CCTGTAGCGG   7980

CGCATTAAGC GCGGCGGGTG TGGTGGTTAC GCGCAGCGTG ACCGCTACAC TTGCCAGCGC   8040

CCTAGCGCCC GCTCCTTTCG CTTTCTTCCC TTCCTTTCTC GCCACGTTCG CCGGCTTTCC   8100

CCGTCAAGCT CTAAATCGGG GGCTCCCTTT AGGGTTCCGA TTTAGTGCTT TACGGCACCT   8160

CGACCCCAAA AAACTTGATT AGGGTGATGG TTCACGTAGT GGGCCATCGC CCTGATAGAC   8220

GGTTTTTCGC CCTTTGACGT TGGAGTCCAC GTTCTTTAAT AGTGGACTCT TGTTCCAAAC   8280

TGGAACAACA CTCAACCCTA TCTCGGTCTA TTCTTTTGAT TTATAAGGGA TTTTGCCGAT   8340

TTCGGCCTAT TGGTTAAAAA ATGAGCTGAT TTAACAAAAA TTTAACGCGA ATTTTAACAA   8400

AATATTAACG TTTACAATTT CCCAGGTGGC ACTTTTCGGG GAAATGTGCG CGGAACCCCT   8460

ATTTGTTTAT TTTTCTAAAT ACATTCAAAT ATGTATCCGC TCATGAGACA ATAACCCTGA   8520

TAAATGCTTC AATAATATTG AAAAAGGAAG AGTATGAGTA TTCAACATTT CCGTGTCGCC   8580

CTTATTCCCT TTTTTGCGGC ATTTTGCCTT CCTGTTTTTG CTCACCCAGA AACGCTGGTG   8640

AAAGTAAAAG ATGCTGAAGA TCAGTTGGGT GCACGAGTGG GTTACATCGA ACTGGATCTC   8700

AACAGCGGTA AGATCCTTGA GAGTTTTCGC CCCGAAGAAC GTTTTCCAAT GATGAGCACT   8760
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TTTAAAGTTC TGCTATGTGG CGCGGTATTA TCCCGTATTG ACGCCGGGCA AGAGCAACTC    8820

GGTCGCCGCA TACACTATTC TCAGAATGAC TTGGTTGAGT ACTCACCAGT CACAGAAAAG    8880

CATCTTACGG ATGGCATGAC AGTAAGAGAA TTATGCAGTG CTGCCATAAC CATGAGTGAT    8940

AACACTGCGG CCAACTTACT TCTGACAACG ATCGGAGGAC CGAAGGAGCT AACCGCTTTT    9000

TTGCACAACA TGGGGGATCA TGTAACTCGC CTTGATCGTT GGGAACCGGA GCTGAATGAA    9060

GCCATACCAA ACGACGAGCG TGACACCACG ATGCCTGTAG CAATGGCAAC AACGTTGCGC    9120

AAACTATTAA CTGGCGAACT ACTTACTCTA GCTTCCCGGC AACAATTAAT AGACTGGATG    9180

GAGGCGGATA AAGTTGCAGG ACCACTTCTG CGCTCGGCCC TTCCGGCTGG CTGGTTTATT    9240

GCTGATAAAT CTGGAGCCGG TGAGCGTGGG TCTCGCGGTA TCATTGCAGC ACTGGGGCCA    9300

GATGGTAAGC CCTCCCGTAT CGTAGTTATC TACACGACGG GGAGTCAGGC AACTATGGAT    9360

GAACGAAATA GACAGATCGC TGAGATAGGT GCCTCACTGA TTAAGCATTG GTAACTGTCA    9420

GACCAAGTTT ACTCATATAT ACTTTAGATT GATTTAAAAC TTCATTTTTA ATTTAAAAGG    9480

ATCTAGGTGA AGATCCTTTT TGATAATCTC ATGACCAAAA TCCCTTAACG TGAGTTTTCG    9540

TTCCACTGAG CGTCAGACCC CGTAGAAAAG ATCAAAGGAT CTTCTTGAGA TCCTTTTTTT    9600

CTGCGCGTAA TCTGCTGCTT GCAAACAAAA AAACCACCGC TACCAGCGGT GGTTTGTTTG    9660

CCGGATCAAG AGCTACCAAC TCTTTTTCCG AAGGTAACTG GCTTCAGCAG AGCGCAGATA    9720

CCAAATACTG TCCTTCTAGT GTAGCCGTAG TTAGGCCACC ACTTCAAGAA CTCTGTAGCA    9780

CCGCCTACAT ACCTCGCTCT GCTAATCCTG TTACCAGTGG CTGCTGCCAG TGGCGATAAG    9840

TCGTGTCTTA CCGGGTTGGA CTCAAGACGA TAGTTACCGG ATAAGGCGCA GCGGTCGGGC    9900

TGAACGGGGG GTTCGTGCAC ACAGCCCAGC TTGGAGCGAA CGACCTACAC CGAACTGAGA    9960

TACCTACAGC GTGAGCTATG AGAAAGCGCC ACGCTTCCCG AAGGGAGAAA GGCGGACAGG   10020

TATCCGGTAA GCGGCAGGGT CGGAACAGGA GAGCGCACGA GGGAGCTTCC AGGGGGAAAC   10080

GCCTGGTATC TTTATAGTCC TGTCGGGTTT CGCCACCTCT GACTTGAGCG TCGATTTTTG   10140

TGATGCTCGT CAGGGGGGCG GAGCCTATGG AAAAACGCCA GCAACGCGGC CTTTTTACGG   10200

TTCCTGGCCT TTTGCTGGCC TTTTGCTCAC ATGTTCTTTC CTGCGTTATC CCCTGATTCT   10260

GTGGATAACC GTATTACCGC CTTTGAGTGA GCTGATACCG CTCGCCGCAG CCGAACGACC   10320

GAGCGCAGCG AGTCAGTGAG CGAGGAAGCG GAAGAGCGCC CAATACGCAA ACCGCCTCTC   10380

CCCGCGCGTT GGCCGATTCA TTAATGCAGC TGGCACGACA GGTTTCCCGA CTGGAAAGCG   10440

GGCAGTGAGC GCAACGCAAT TAATGTGAGT TAGCTCACTC ATTAGGCACC CCAGGCTTTA   10500

CACTTTATGC TTCCGGCTCG TATGTTGTGT GGAATTGTGA GCGGATAACA ATTTCACACA   10560

GGAAACAGCT ATGACCATGA TTACGCCAAG CTTTTGCGAT CAATAAATGG ATCACAACCA   10620

GTATCTCTTA ACGATGTTCT TCGCAGATGA TGATTCATTT TTTAAGTATT TGGCTAGTCA   10680

AGATGATGAA TCTTCATTAT CTGATATATT GCAAATCACT CAATATCTAG ACTTTCTGTT   10740

ATTATTATTG ATCCAATCAA AAAATAAATT AGAAGCCGTG GGTCATTGTT ATGAATCTCT   10800

TTCAGAGGAA TACAGACAAT TGACAAAATT CACAGACTTT CAAGATTTTA AAAAACTGTT   10860
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TAACAAGGTC CCTATTGTTA CAGATGGAAG GGTCAAACTT AATAAAGGAT ATTTGTTCGA    10920

CTTTGTGATT AGTTTGATGC GATTCAAAAA AGAATCCTCT CTAGCTACCA CCGCAATAGA    10980

TCCTGTTAGA TACATAGATC CTCGTCGCAA TATCGCATTT TCTAACGTGA TGGATATATT    11040

AAAGTCGAAT AAAGTGAACA ATAATTAATT CTTTATTGTC ATCATGAACG GCGGACATAT    11100

TCAGTTGATA ATCGGCCCCA TGTTTTCAGG TAAAAGTACA GAATTAATTA GACGAGTTAG    11160

ACGTTATCAA ATAGCTCAAT ATAAATGCGT GACTATAAAA TATTCTAACG ATAATAGATA    11220

CGGAACGGGA CTATGGACGC ATGATAAGAA TAATTTTGAA GCATTGGAAG CAACTAAACT    11280

ATGTGATGTC TTGGAATCAA TTACAGATTT CTCCGTGATA GGTATCGATG AAGGACAGTT    11340

CTTTCCAGAC ATTGTTGAAT TGATCTCGAT CCCGCGAAAT TAATACGACT CACTATAGGG    11400

AGACCACAAC GGTTTCCCTC TAGCGGGATC AATTCCGCCC CTCTCCCTCC CCCCCCCCTA    11460

ACGTTACTGG CCGAAGCCGC TTGGAATAAG GCCGGTGTGC GTTTGTCTAT ATGTTATTTT    11520

CCACCATATT GCCGTCTTTT GGCAATGTGA GGGCCCGGAA ACCTGGCCCT GTCTTCTTGA    11580

CGAGCATTCC TAGGGGTCTT TCCCCTCTCG CCAAAGGAAT GCAAGGTCTG TTGAATGTCG    11640

TGAAGGAAGC AGTTCCTCTG GAAGCTTCTT GAAGACAAAC AACGTCTGTA GCGACCCTTT    11700

GCAGGCAGCG GAACCCCCCA CCTGGCGACA GGTGCCTCTG CGGCCAAAAG CCACGTGTAT    11760

AAGATACACC TGCAAAGGCG GCACAACCCC AGTGCCACGT TGTGAGTTGG ATAGTTGTGG    11820

AAAGAGTCAA ATGGCTCTCC TCAAGCGTAT TCAACAAGGG GCTGAAGGAT GCCCAGAAGG    11880

TACCCCATTG TATGGGATCT GATCTGGGGC CTCGGTGCAC ATGCTTTACA TGTGTTTAGT    11940

CGAGGTTAAA AAACGTCTAG GCCCCCCGAA CCACGGGGAC GTGGTTTTCC TTTGAAAAAC    12000

ACGATAATAC C                                                        12011
```

(2) INFORMATION FOR SEQ ID NO:23:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 15 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: cDNA

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

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GATCGATGCT AGCCC                                                      15
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(2) INFORMATION FOR SEQ ID NO:24:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 15 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: cDNA

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GATCGGGCTA GCATC   15

(2) INFORMATION FOR SEQ ID NO:25:

          (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 12 base pairs
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

         (ii) MOLECULE TYPE: cDNA

         (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

TTACATGGCC AT   12

(2) INFORMATION FOR SEQ ID NO:26:

          (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 12 base pairs
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

         (ii) MOLECULE TYPE: cDNA

         (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

TCACATGGCG AT   12

(2) INFORMATION FOR SEQ ID NO:27:

          (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 12 base pairs
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

         (ii) MOLECULE TYPE: cDNA

         (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TTTGGACTGG GC   12

(2) INFORMATION FOR SEQ ID NO:28:

          (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 12 base pairs
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

         (ii) MOLECULE TYPE: cDNA

         (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

TTTTGATTGG GC   12

(2) INFORMATION FOR SEQ ID NO:29:

          (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 14 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

TGGTCCTAAT ACTG   14

(2) INFORMATION FOR SEQ ID NO:30:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 14 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

TGGGCCTAAT ATCG   14

(2) INFORMATION FOR SEQ ID NO:31:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 30 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GCATTATCTA GATGTGTCTT CTGGTCAGAG   30

(2) INFORMATION FOR SEQ ID NO:32:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 30 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CCTGAATTAT AATAATTAAC TGCAGGTCCT   30

(2) INFORMATION FOR SEQ ID NO:33:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 26 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

GGGAAAGAAT CCAGAGACAA GAACGG   26

(2) INFORMATION FOR SEQ ID NO:34:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 26 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

GGTGAAGTTG TGGATCCATT TGATTG   26

(2) INFORMATION FOR SEQ ID NO:35:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 24 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

CAACCTGTAA GGTACCAGCA TCCG   24

(2) INFORMATION FOR SEQ ID NO:36:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 27 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

GATATGGTGT TAGGCCTTGA TCTGTTC   27

(2) INFORMATION FOR SEQ ID NO:37:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 29 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

CGCCATGGAA AAATCAGAGA TCCTCTTCT   29

(2) INFORMATION FOR SEQ ID NO:38:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 31 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

CTGGATCCTA ATTGGAGTTG TTACCCATGT A   31

(2) INFORMATION FOR SEQ ID NO:39:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 23 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

AACCATGGCT GAAAAGGGA AAA   23

(2) INFORMATION FOR SEQ ID NO:40:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 33 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

GGTGAAGCTT AAGATGTGAT TTTACATATT TTA   33

(2) INFORMATION FOR SEQ ID NO:41:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 35 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

AAATAGGATC CCTACAGATC ATTAGATATT AAAAT   35

(2) INFORMATION FOR SEQ ID NO:42:

    (i) SEQUENCE CHARACTERISTICS:

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                    (A)  LENGTH: 24 base pairs
                    (B)  TYPE: nucleic acid
                    (C)  STRANDEDNESS: single
                    (D)  TOPOLOGY: linear

              (ii) MOLECULE TYPE: cDNA


              (xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

CGCCATGGTG TTCAGTGCTT GTTG                                            24

(2) INFORMATION FOR SEQ ID NO:43:

              (i)  SEQUENCE CHARACTERISTICS:
                    (A)  LENGTH: 31 base pairs
                    (B)  TYPE: nucleic acid
                    (C)  STRANDEDNESS: single
                    (D)  TOPOLOGY: linear

              (ii) MOLECULE TYPE: cDNA


              (xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

CCACAAGCTT AATTAACCAT AATATGCATC A                                    31

(2) INFORMATION FOR SEQ ID NO:44:

              (i)  SEQUENCE CHARACTERISTICS:
                    (A)  LENGTH: 29 base pairs
                    (B)  TYPE: nucleic acid
                    (C)  STRANDEDNESS: single
                    (D)  TOPOLOGY: linear

              (ii) MOLECULE TYPE: cDNA


              (xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

TTCCATGGAT TTGGATTTGT CTATTGGGT                                       29

(2) INFORMATION FOR SEQ ID NO:45:

              (i)  SEQUENCE CHARACTERISTICS:
                    (A)  LENGTH: 15462 base pairs
                    (B)  TYPE: nucleic acid
                    (C)  STRANDEDNESS: single
                    (D)  TOPOLOGY: linear

              (ii) MOLECULE TYPE: cDNA


              (xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

ACCAAACAAG AGAAGAAACT TGCTTGGTAA TATAAATTTA ACTTAAAATT AACTTAGGAT     60

TTAAGACATT GACTAGAAGG TCAAGAAAAG GGAACTCTAT AATTTCAAAA ATGTTGAGCC    120

TATTTGATAC ATTTAATGCA CGTAGGCAAG AAAACATAAC AAAATCAGCC GGTGGAGCTA    180

TCATTCCTGG ACAGAAAAAT ACTGTCTCTA TATTCGCCCT TGGACCGACA ATAACTGATG    240

ATAATGAGAA AATGACATTA GCTCTTCTAT TTCTATCTCA TTCACTAGAT AATGAGAAAC    300
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AACATGCACA AAGGGCAGGG TTCTTGGTGT CTTTATTGTC AATGGCTTAT GCCAATCCAG    360

AGCTCTACCT AACAACAAAT GGAAGTAATG CAGATGCCAA GTATGTCATA TACATGATTG    420

AGAAAGATCT AAAACGGCAA AAGTATGGAG GATTTGTGGT TAAGACGAGA GAGATGATAT    480

ATGAAAAGAC AACTGATTGG ATATTTGGAA GTGACCTGGA TTATGATCAG GAAACTATGT    540

TGCAGAACGG CAGGAACAAT TCAACAATTG AAGACCTTGT CCACACATTT GGGTATCCAT    600

CATGTTTAGG AGCTCTTATA ATACAGATCT GGATAGTTCT GGTCAAAGCT ATCACTAGTA    660

TCTCAGGGTT AAGAAAAGGC TTTTTCACCC GATTGGAAGC TTTCAGACAA GATGGAACAG    720

TGCAGGCAGG GCTGGTATTG AGCGGTGACA CAGTGGATCA GATTGGGTCA ATCATGCGGT    780

CTCAACAGAG CTTGGTAACT CTTATGGTTG AAACATTAAT AACAATGAAT ACCAGCAGAA    840

ATGACCTCAC AACCATAGAA AAGAATATAC AAATTGTTGG CAACTACATA AGAGATGCAG    900

GTCTCGCTTC ATTCTTCAAT ACAATCAGAT ATGGAATTGA GACCAGAATG GCAGCTTTGA    960

CTCTATCCAC TCTCAGACCA GATATCAATA GATTAAAAGC TTTGATGGAA CTGTATTTAT   1020

CAAAGGGACC ACGCGCTCCT TTCATCTGTA TCCTCAGAGA TCCTATACAT GGTGAGTTCG   1080

CACCAGGCAA CTATCCTGCC ATATGGAGCT ATGCAATGGG GGTGGCAGTT GTACAAAATA   1140

GAGCCATGCA ACAGTATGTG ACGGGAAGAT CATATCTAGA CATTGATATG TTCCAGCTAG   1200

GACAAGCAGT AGCACGTGAT GCCGAAGCTC AAATGAGCTC AACACTGGAA GATGAACTTG   1260

GAGTGACACA CGAAGCTAAA GAAAGCTTGA AGAGACATAT AAGGAACATA AACAGTTCAG   1320

AGACATCTTT CCACAAACCG ACAGGTGGAT CAGCCATAGA GATGGCAATA GATGAAGAGC   1380

CAGAACAATT CGAACATAGA GCAGATCAAG AACAAAATGG AGAACCTCAA TCATCCATAA   1440

TTCAATATGC CTGGGCAGAA GGAAATAGAA GCGATGATCA GACTGAGCAA GCTACAGAAT   1500

CTGACAATAT CAAGACCGAA CAACAAAACA TCAGAGACAG ACTAAACAAG AGACTCAACG   1560

ACAAGAAGAA ACAAAGCAGT CAACCACCCA CTAATCCCAC AAACAGAACA AACCAGGACG   1620

AAATAGATGA TCTGTTTAAC GCATTTGGAA GCAACTAATC GAATCAACAT TTTAATCTAA   1680

ATCAATAATA AATAAGAAAA ACTTAGGATT AAAGAATCCT ATCATACCGG AATATAGGGT   1740

GGTAAATTTA GAGTCTGCTT GAAACTCAAT CAATAGAGAG TTGATGGAAA GCGATGCTAA   1800

AAACTATCAA ATCATGGATT CTTGGGAAGA GGAATCAAGA GATAAATCAA CTAATATCTC   1860

CTCGGCCCTC AACATCATTG AATTCATACT CAGCACCGAC CCCCAAGAAG ACTTATCGGA   1920

AAACGACACA ATCAACACAA GAACCCAGCA ACTCAGTGCC ACCATCTGTC AACCAGAAAT   1980

CAAACCAACA GAAACAAGTG AGAAAGATAG TGGATCAACT GACAAAAATA GACAGTCTGG   2040

GTCATCACAC GAATGTACAA CAGAAGCAAA AGATAGAAAC ATTGATCAGG AAACTGTACA   2100

GAGAGGACCT GGGAGAAGAA GCAGCTCAGA TAGTAGAGCT GAGACTGTGG TCTCTGGAGG   2160

AATCCCCAGA AGCATCACAG ATTCTAAAAA TGGAACCCAA AACACGGAGG ATATTGATCT   2220

CAATGAAATT AGAAAGATGG ATAAGGACTC TATTGAGGGG AAAATGCGAC AATCTGCAAA   2280

TGTTCCAAGC GAGATATCAG GAAGTGATGA CATATTTACA ACAGAACAAA GTAGAAACAG   2340

TGATCATGGA AGAAGCCTGG AATCTATCAG TACACCTGAT ACAAGATCAA TAAGTGTTGT   2400
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TACTGCTGCA ACACCAGATG ATGAAGAAGA AATACTAATG AAAAATAGTA GGACAAAGAA    2460

AAGTTCTTCA ACACATCAAG AAGATGACAA AAGAATTAAA AAAGGGGGAA AAGGGAAAGA    2520

CTGGTTTAAG AAATCAAAAG ATACCGACAA CCAGATACCA ACATCAGACT ACAGATCCAC    2580

ATCAAAAGGG CAGAAGAAAA TCTCAAAGAC AACAACCACC AACACCGACA CAAAGGGGCA    2640

AACAGAAATA CAGACAGAAT CATCAGAAAC ACAATCCTCA TCATGGAATC TCATCATCGA    2700

CAACAACACC GACCGGAACG AACAGACAAG CACAACTCCT CCAACAACAA CTTCCAGATC    2760

AACTTATACA AAAGAATCGA TCCGAACAAA CTCTGAATCC AAACCCAAGA CACAAAAGAC    2820

AAATGGAAAG GAAAGGAAGG ATACAGAAGA GAGCAATCGA TTTACAGAGA GGGCAATTAC    2880

TCTATTGCAG AATCTTGGTG TAATTCAATC CACATCAAAA CTAGATTTAT ATCAAGACAA    2940

ACGAGTTGTA TGTGTAGCAA ATGTACTAAA CAATGTAGAT ACTGCATCAA AGATAGATTT    3000

CCTGGCAGGA TTAGTCATAG GGGTTTCAAT GGACAACGAC ACAAAATTAA CACAGATACA    3060

AAATGAAATG CTAAACCTCA AAGCAGATCT AAAGAAAATG GACGAATCAC ATAGAAGATT    3120

GATAGAAAAT CAAAGAGAAC AACTGTCATT GATCACGTCA CTAATTTCAA ATCTCAAAAT    3180

TATGACTGAG AGAGGAGGAA AGAAAGACCA AAATGAATCC AATGAGAGAG TATCCATGAT    3240

CAAAACAAAA TTGAAAGAAG AAAAGATCAA GAAGACCAGG TTTGACCCAC TTATGGAGGC    3300

ACAAGGCATT GACAAGAATA TACCCGATCT ATATCGACAT GCAGGAGATA CACTAGAGAA    3360

CGATGTACAA GTTAAATCAG AGATATTAAG TTCATACAAT GAGTCAAATG CAACAAGACT    3420

AATACCCAAA AAAGTGAGCA GTACAATGAG ATCACTAGTT GCAGTCATCA ACAACAGCAA    3480

TCTCTCACAA AGCACAAAAC AATCATACAT AAACGAACTC AAACGTTGCA AAAATGATGA    3540

AGAAGTATCT GAATTAATGG ACATGTTCAA TGAAGATGTC AACAATTGCC AATGATCCAA    3600

CAAAGAAACG ACACCGAACA AACAGACAAG AAACAACAGT AGATCAAAAC CTGTCAACAC    3660

ACACAAAATC AAGCAGAATG AAACAACAGA TATCAATCAA TATACAAATA AGAAAAACTT    3720

AGGATTAAAG AATAAATTAA TCCTTGTCCA AAATGAGTAT AACTAACTCT GCAATATACA    3780

CATTCCCAGA ATCATCATTC TCTGAAAATG GTCATATAGA ACCATTACCA CTCAAAGTCA    3840

ATGAACAGAG GAAAGCAGTA CCCCACATTA GAGTTGCCAA GATCGGAAAT CCACCAAAAC    3900

ACGGATCCCG GTATTTAGAT GTCTTCTTAC TCGGCTTCTT CGAGATGGAA CGAATCAAAG    3960

ACAAATACGG GAGTGTGAAT GATCTCGACA GTGACCCGAG TTACAAAGTT TGTGGCTCTG    4020

GATCATTACC AATCGGATTG GCTAAGTACA CTGGGAATGA CCAGGAATTG TTACAAGCCG    4080

CAACCAAACT GGATATAGAA GTGAGAAGAA CAGTCAAAGC GAAAGAGATG GTTGTTTACA    4140

CGGTACAAAA TATAAAACCA GAACTGTACC CATGGTCCAA TAGACTAAGA AAAGGAATGC    4200

TGTTCGATGC CAACAAAGTT GCTCTTGCTC CTCAATGTCT TCCACTAGAT AGGAGCATAA    4260

AATTTAGAGT AATCTTCGTG AATTGTACGG CAATTGGATC AATAACCTTG TTCAAAATTC    4320

CTAAGTCAAT GGCATCACTA TCGTTAACCA ACACAATATC AATCAATCTG CAGGTACACA    4380

TAAAAACAGG GGTTCAGACT GATTCTAAAG GGATAGTTCA AATTTTGGAT GAGAAAGGCG    4440

AAAAATCACT GAATTTCATG GTCCATCTCG GATTGATCAA AAGAAAAGTA GGCAGAATGT    4500
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ACTCTGTTGA ATACTGTAAA CAGAAAATCG AGAAAATGAG ATTGATATTT TCTTTAGGAC    4560

TAGTTGGAGG AATCAGTCTT CATGTCAATG CAACTGGGTC CATATCAAAA ACACTAGCAA    4620

GTCAGCTGGT ATTCAAAAGA GAGATTTGTT ATCCTTTAAT GGATCTAAAT CCGCATCTCA    4680

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CTTTACCTGG CGAGTTCAGA TACTATCCTA ATATTATTGC AAAAGGAGTT GGGAAAATCA    4800

AACAATGGAA CTAGTAATCT CTATTTTAGT CCGGACGTAT CTATTAAGCC GAAGCAAATA    4860

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TCGCAAGAAT AAGAGAGAAG GGACCAAAAA AGTCAAATAG GAGAAATCAA AACAAAAGGT    4980

ACAGAACACC AGAACAACAA AATCAAAACA TCCAACTCAC TCAAAACAAA AATTCCAAAA    5040

GAGACCGGCA ACACAACAAG CACTGAACAC AATGCCAACT TCAATACTGC TAATTATTAC    5100

AACCATGATC ATGGCATCTT TCTGCCAAAT AGATATCACA AAACTACAGC ACGTAGGTGT    5160

ATTGGTCAAC AGTCCCAAAG GGATGAAGAT ATCACAAAAC TTTGAAACAA GATATCTAAT    5220

TTTGAGCCTC ATACCAAAAA TAGAAGACTC TAACTCTTGT GGTGACCAAC AGATCAAGCA    5280

ATACAAGAAG TTATTGGATA GACTGATCAT CCCTTTATAT GATGGATTAA GATTACAGAA    5340

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AATTAGGGAC ACAAACAAAG CAGTGCAGTC AGTTCAGAGC TCCATAGGAA ATTTAATAGT    5580

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CACTCAGATC TACAAAGTAG ATTCCATATC ATATAACATC AAAACAGAG AATGGTATAT    6000

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TGAAATAGAG AGCTGCTTAT CAGGAAACAT ATCCCAATGT CCAAGAACAA CGGTCACATC    6180

AGACATTGTT CCAAGATATG CATTTGTCAA TGGAGGAGTG GTTGCAAACT GTATAACAAC    6240

CACCTGTACA TGCAACGGAA TTGGTAATAG AATCAATCAA CCACCTGATC AAGGAGTAAA    6300

AATTATAACA CATAAAGAAT GTAGTACTGT GGGTATCAAC GGAATGCTGT TCAATACAAA    6360

TAAAGAAGGA ACTCTTGCAT TCTATACACC AAATGATATA ACACTAAACA ATTCTGTTAC    6420

ACTGGATCCA ATTGACATAT CAATCGAGCT CAACAAGGCC AAATCAGATC TAGAAGAATC    6480

AAAAGAATGG ATAAGAAGGT CAAATCAAAA ACTAGATTCT ATTGGAAATT GGCATCAATC    6540

TAGCACTACA ATCATAATTA TTTTGATAAT GATCATTATA TTGTTTATAA TTAATATAAC    6600
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178

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GATAATTACA ATTGCAATTA AGTATTACAG AATTCAAAAG AGAAATCGAG TGGATCAAAA   6660

TGACAAGCCA TATGTACTAA CAAACAAATA ACATATCTAC AGATCATTAG ATATTAAAAT   6720

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CCCAATAGAC AAATCCAAAT TCGAGATGGA ATACTGGAAG CATACCAATC ACGGAAAGGA   6840

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ATCATTGACA CAACAGATGT CAGATCTTAG GAAATTCATT AGTGAAATTA CAATTAGAAA   7200

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TGTCAATTAT GATGGTTCAA TCTCAACAAC AAGATTTAAG AATAATAACA TAAGCTTTGA   7740

TCAACCATAT GCTGCACTAT ACCCATCTGT TGGACCAGGG ATATACTACA AAGGCAAAAT   7800

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ACAATTAGGA ATAATTGATA TTACTGATTA CAGTGATATA AGGATAAAAT GGACATGGCA   8160

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TTAATCATAA TTAACCATAA TATGCATCAA TCTATCTATA ATACAAGTAT ATGATAAGTA   8580

ATCAGCAATC AGACAATAGA CAAAAGGGAA ATATAAAAAA CTTAGGAGCA AAGCGTGCTC   8640

GGGAAATGGA CACTGAATCT AACAATGGCA CTGTATCTGA CATACTCTAT CCTGAGTGTC   8700
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ACCTTAACTC TCCTATCGTT AAAGGTAAAA TAGCACAATT ACACACTATT ATGAGTCTAC   8760

CTCAGCCTTA TGATATGGAT GACGACTCAA TACTAGTTAT CACTAGACAG AAAATAAAAC   8820

TTAATAAATT GGATAAAAGA CAACGATCTA TTAGAAGATT AAAATTAATA TTAACTGAAA   8880

AAGTGAATGA CTTAGGAAAA TACACATTTA TCAGATATCC AGAAATGTCA AAAGAAATGT   8940

TCAAATTATA TATACCTGGT ATTAACAGTA AAGTGACTGA ATTATTACTT AAAGCAGATA   9000

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TAGCCTCAAA AAATGATGGA AGCAATTATG ATCTTAATGA AGAAATTAAT AATATATCGA   9120

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TCAAGTATGA TATGAGAAGA TTACAAAAAG CTCGAAATGA GATCACTTTT AATGTTGGGA   9240

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ATCAGATATT GGATTATGTA GAATCTGGGG ACTGGTTAGA TGATCCAGAA TTTAATATTT  10260

CTTATAGTCT TAAAGAAAAA GAGATCAAAC AGGAAGGTAG ACTCTTTGCA AAAATGACAT  10320

ACAAAATGAG AGCTACACAA GTTTTATCAG AGACCCTACT TGCAAATAAC ATAGGAAAAT  10380

TCTTTCAAGA AAATGGGATG GTGAAGGGAG AGATTGAATT ACTTAAGAGA TTAACAACCA  10440

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TCCTAACAAC AGATCTCAAA AAATACTGTC TTAATTGGAG ATATGAATCA ACAGCTCTAT  10680

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GTCTTGAAGG AAGTACAATC TATGTAGGTG ATCCTTACTG TCCTCCATCA GATAAAGAAC  10800
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AATTAAATGA AACGATTATA AGTAGCAAGA TGTTCATATA TAGCAAAAGA ATCTATTATG   11160

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TTGCACATGA TATTCTAGAT AATTCTCTCA CAGGAATTAG AAATGCCATA GCTGGAATGT   11880

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TGTTGAGGAA AATCAGTAAT TACGATCTAG TACAATATGA AACACTAAGT AGGACTTTGC   12000

GACTAATTGT AAGTGATAAA ATCAAGTATG AAGATATGTG TTCGGTAGAC CTTGCCATAG   12060

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CGCCTGACCC ATTAGAATTA CTATCTGGGG TAGTAATAAC AGGATCAGAA CATTGTAAAA   12180

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CTGATGAAAG ATCTGAAGCA CAATTAGGAT ATATCAAGAA TCTTAGTAAA CCTGCAAAAG   12360

CCGCAATAAG AATAGCAATG ATATATACAT GGGCATTTGG TAATGATGAG ATATCTTGGA   12420

TGGAAGCCTC ACAGATAGCA CAAACACGTG CAAATTTTAC ACTAGATAGT CTCAAAATTT   12480

TAACACCGGT AGCTACATCA ACAAATTTAT CACACAGATT AAAGGATACT GCAACTCAGA   12540

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ATGAACATAT TAATCCAGAG TCTACATTAG AATTAATTCG ATATCCTGAA AGTAATGAAT   12840

TTATTTATGA TAAAGACCCA CTCAAAGATG TGGACTTATC AAAACTTATG GTTATTAAAG   12900
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ACCATTCTTA CACAATTGAT ATGAATTATT GGGATGATAC TGACATCATA CATGCAATTT   12960

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TATTGAAACA ATATCTTGAA TTAAATATTA AGAAGACCC TACTCTTAAA TATGTACAAA    13620

TATCTGGATT ATTAATTAAA TCGTTCCCAT CAACTGTAAC ATACGTAAGA AAGACTGCAA   13680

TCAAATATCT AAGGATTCGC GGTATTAGTC CACCTGAGGT AATTGATGAT TGGGATCCGG   13740

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CATGGATAGG AAATATGGAA TGTGAGAGCT TAATATGGAG TGAATTAAAT GATAAGTCCA   14340

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CAAAGAAGAG GAATAATGAA TGGTTACATC ATGAAATCAA AGAAGGAGAA AGAGATTATG   14760

GAATCATGAG ACCATATCAT ATGGCACTAC AAATCTTTGG ATTTCAAATC AATTTAAATC   14820

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GTTTTCAGCG AACAATAAAG GATGTTTTAT TTGAATGGAT TAATATAACT CATGATGATA   14940

AGAGACATAA ATTAGGCGGA AGATATAACA TATTCCCACT GAAAAATAAG GGAAAGTTAA   15000
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GACTGCTATC GAGAAGACTA GTATTAAGTT GGATTTCATT ATCATTATCG ACTCGATTAC   15060

TTACAGGTCG CTTTCCTGAT GAAAAATTTG AACATAGAGC ACAGACTGGA TATGTATCAT   15120

TAGCTGATAC TGATTTAGAA TCATTAAAGT TATTGTCGAA AAACATCATT AAGAATTACA   15180

GAGAGTGTAT AGGATCAATA TCATATTGGT TTCTAACCAA AGAAGTTAAA ATACTTATGA   15240

AATTGATCGG TGGTGCTAAA TTATTAGGAA TTCCCAGACA ATATAAAGAA CCCGAAGACC   15300

AGTTATTAGA AAACTACAAT CAACATGATG AATTTGATAT CGATTAAAAC ATAAATACAA   15360

TGAAGATATA TCCTAACCTT TATCTTTAAG CCTAGGAATA GACAAAAAGT AAGAAAAACA   15420

TGTAATATAT ATATACCAAA CAGAGTTCTT CTCTTGTTTG GT   15462

(2) INFORMATION FOR SEQ ID NO:46:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 12 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

TTGTCTGGGA AT   12

(2) INFORMATION FOR SEQ ID NO:47:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 12 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

TTGCCTGGGA AT   12

(2) INFORMATION FOR SEQ ID NO:48:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 12 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

TTGTTTGGGA AT   12

(2) INFORMATION FOR SEQ ID NO:49:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 12 base pairs
        (B) TYPE: nucleic acid

            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

TTGTCTGGTA AT   12

(2) INFORMATION FOR SEQ ID NO:50:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 12 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

AACTTTAAAT TA   12

(2) INFORMATION FOR SEQ ID NO:51:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 12 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

AACTTAAAAT TA   12

(2) INFORMATION FOR SEQ ID NO:52:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 12 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

TTAAAGACAT TG   12

(2) INFORMATION FOR SEQ ID NO:53:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 12 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

TTTAAGACAT TG   12

(2) INFORMATION FOR SEQ ID NO:54:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 12 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

GCAGATGTCA AG   12

(2) INFORMATION FOR SEQ ID NO:55:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 12 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

GCAGATGCCA AG   12

(2) INFORMATION FOR SEQ ID NO:56:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 12 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

CGAATCTAAA GA   12

(2) INFORMATION FOR SEQ ID NO:57:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 12 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

CGAAGCTAAA GA   12

(2) INFORMATION FOR SEQ ID NO:58:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 12 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

GAAATATTGA TC   12

(2) INFORMATION FOR SEQ ID NO:59:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 12 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

GAAACATTGA TC   12

(2) INFORMATION FOR SEQ ID NO:60:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 12 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

TCTCTACCCA AC   12

(2) INFORMATION FOR SEQ ID NO:61:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 12 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

TCGTTAACCA AC   12

(2) INFORMATION FOR SEQ ID NO:62:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 12 base pairs
       (B) TYPE: nucleic acid

        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

AGTACAATAG GT   12

(2) INFORMATION FOR SEQ ID NO:63:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 12 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

AGTACTGTGG GT   12

(2) INFORMATION FOR SEQ ID NO:64:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 12 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

GCACTTGATC CA   12

(2) INFORMATION FOR SEQ ID NO:65:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 12 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

ACACTGGATC CA   12

(2) INFORMATION FOR SEQ ID NO:66:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 19 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

CCATCATTGT TGTTGACAA   19

(2) INFORMATION FOR SEQ ID NO:67:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 19 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

CCATCATTGT GGCTGACAA   19

(2) INFORMATION FOR SEQ ID NO:68:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 11 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

TTACATGGCC A   11

(2) INFORMATION FOR SEQ ID NO:69:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 11 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

TCACATGGCG A   11

(2) INFORMATION FOR SEQ ID NO:70:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 12 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

TTTGGACTGG GC   12

(2) INFORMATION FOR SEQ ID NO:71:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 12 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

TTTTGATTGG GC   12

(2) INFORMATION FOR SEQ ID NO:72:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 12 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:72:

GGTCCTAATA CT   12

(2) INFORMATION FOR SEQ ID NO:73:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 12 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:

GGGCCTAATA TC   12

(2) INFORMATION FOR SEQ ID NO:74:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 38 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:74

CCATAGAGAG TCCATGGAAA GCGACGCTAA AAACTATC   38

WHAT IS CLAIMED IS:

        1.   An isolated polynucleotide molecule comprising
an operably linked transcriptional promoter, a polynucleotide
sequence encoding a PIV genome or antigenome, and a
transcriptional terminator, wherein said polynucleotide
sequence encoding said PIV genome or antigenome is modified by
introduction of a heterologous PIV sequence selected from a
HPIV1 sequence, a HPIV2 sequence, a HPIV3 sequence, a BPIV
sequence or a MPIV sequence to form a chimeric PIV genome or
antigenome.

        2.   The isolated polynucleotide molecule of claim
1, wherein a gene or gene segment of human PIV3 is replaced
with a counterpart gene or gene segment from a heterologous
PIV.

        3.   The isolated polynucleotide molecule of claim
2, wherein the counterpart gene or gene segment is a HN or F
glycoprotein gene or gene segment of HPIV1 or HPIV2.

        4.   The isolated polynucleotide molecule of claim
2, wherein an HN or F glycoprotein gene of PIV1 or PIV2 is
substituted for the counterpart HN or F glycoprotein gene of
HPIV3.

        5.   The isolated polynucleotide molecule of claim
1, wherein the polynucleotide sequence encoding the genome or
antigenome incorporates a BPIV gene or gene segment.

        6.   The isolated polynucleotide molecule of claim
1, which incorporates a heterologous sequence from RSV.

        7.   The isolated polynucleotide molecule of claim
6, wherein the heterologous sequence from RSV is a G or F gene
or gene segment.

8.   The isolated polynucleotide molecule of claim
1, which incorporates a heterologous sequence from measles
virus.

9.   The isolated polynucleotide molecule of claim
8, wherein the heterologous sequence from measles virus is a
HA or F gene or gene segment.

10.   An isolated polynucleotide molecule comprising
an operably linked transcriptional promoter, a polynucleotide
sequence encoding a PIV genome or antigenome, and a
transcriptional terminator, wherein said polynucleotide
sequence encoding said PIV genome or antigenome is selected
from the group consisting of:
         i)   p218(131) (SEQ ID NO: 1);
         ii)   p3/7(131) (SEQ ID NO: 14);
         iii)   p3/7(131)2G (SEQ ID NO: 15); or
         iv)   the isolated polynucleotide of i), ii) or iii)
modified by introduction of a heterologous PIV sequence
selected from a HPIV1 sequence, a HPIV2 sequence, a BPIV
sequence or a MPIV sequence or by a nucleotide insertion,
rearrangement, deletion or substitution specifying a
phenotypic alteration selected from attenuation, temperature-
sensitivity, cold-adaptation, small plaque size, host range
restriction, or a change in an immunogenic epitope of PIV.

11.   An isolated polynucleotide molecule comprising
an operably linked transcriptional promoter, a polynucleotide
sequence encoding a PIV genome or antigenome, and a
transcriptional terminator, wherein said polynucleotide
sequence encoding said PIV genome or antigenome is modified by
a nucleotide insertion, rearrangement, deletion or
substitution.

12.   The isolated polynucleotide molecule of claim
11, wherein said nucleotide insertion, rearrangement, deletion
or substitution specifies a phenotypic alteration selected
from attenuation, temperature-sensitivity, cold-adaptation,

small plaque size, host range restriction, or a change in an
immunogenic epitope of PIV.

13. The isolated polynucleotide molecule of claim
12, wherein said polynucleotide sequence encoding said PIV
genome or antigenome incorporates multiple *ts* mutations.

14. The isolated polynucleotide molecule of claim
12, wherein said polynucleotide sequence encoding said PIV
genome or antigenome incorporates multiple non-*ts* attenuating
mutations.

15. The isolated polynucleotide molecule of claim
11, wherein said polynucleotide sequence encoding said PIV
genome or antigenome incorporates one or more mutations of JS
*cp*45.

16. The isolated polynucleotide molecule of claim
15, wherein said polynucleotide sequence encoding said PIV
genome or antigenome encodes at least one amino acid
substitution in the polymerase L protein.

17. The isolated polynucleotide molecule of claim
16, wherein the amino acid substitution in the polymerase L
protein occurs at a position corresponding to Tyr_{942}, Leu_{992}, or
Thr_{1558} of JS *cp*45.

18. The isolated polynucleotide molecule of claim
11, wherein said polynucleotide sequence encoding said PIV
genome or antigenome encodes at least one amino acid
substitution in the N protein.

19. The isolated polynucleotide molecule of claim
28, wherein the amino acid substitution in the N protein
occurs at a position corresponding to residues Val_{96} or Ser_{389}
of JS *cp*45.

20.   The isolated polynucleotide molecule of claim
11, wherein said polynucleotide sequence encoding said PIV
genome or antigenome encodes an amino acid substitution in the
C protein.

5   21.   The isolated polynucleotide molecule of claim
20, wherein the amino acid substitution in the C protein
occurs at a position corresponding to Ile_{96} of JS cp45.

22.   The isolated polynucleotide molecule of claim
11, wherein said polynucleotide sequence encoding said PIV
10   genome or antigenome encodes at least one amino acid
substitution in the F protein.

23.   The isolated polynucleotide molecule of claim
22, wherein the amino acid substitution in the F protein
occurs at a position corresponding to Ile_{420} or Ala_{450} of JS
15   cp45.

24.   The isolated polynucleotide molecule of claim
11, wherein said polynucleotide sequence encoding said PIV
genome or antigenome encodes an amino acid substitution in the
HN protein.

20   25.   The isolated polynucleotide molecule of claim
24, wherein the amino acid substitution in the HN protein
occurs at a position corresponding to residue Val_{384} of JS
cp45.

26.   The isolated polynucleotide molecule of claim
25   11, wherein said polynucleotide sequence encoding said PIV
genome or antigenome incorporates at least one mutation in a
3' leader sequence.

27.   The isolated polynucleotide molecule of claim
26, wherein the mutation in the 3' leader occurs at a position
30   corresponding to nucleotide 23, 24, 28, or 45 of JS cp45.

28. The isolated polynucleotide molecule of claim
11, wherein said polynucleotide sequence encoding said PIV
genome or antigenome incorporates a mutation in a N gene start
sequence.

5   29. The isolated polynucleotide molecule of claim
28, wherein the mutation in the N gene start sequence occurs
at a position corresponding to nucleotide 62 of JS *cp*45.

30. The isolated polynucleotide molecule of claim
12, wherein said polynucleotide sequence encoding said PIV
10   genome or antigenome incorporates a plurality and up to a full
complement of mutations present in r*cp*45, r*cp*45 3'NCMFHN,
r*cp*45 3'NL, r*cp*45 3'N, or r*cp*45 F.

31. The isolated polynucleotide molecule of claim
12, which is an antigenomic cDNA selected from r*cp*45, r*cp*45
15   3'NCMFHN, r*cp*45 3'NL, r*cp*45 3'N, r*cp*45 L, r*cp*45 F, r*cp*45 M,
r*cp*45 HN, or r*cp*45 C.

32. The isolated polynucleotide molecule of claim
11, wherein said polynucleotide sequence encoding said PIV
genome or antigenome incorporates a mutation stabilized by
20   multiple nucleotide substitutions in a codon specifying the
mutation.

33. The isolated polynucleotide molecule of claim
11, wherein said polynucleotide sequence encoding said PIV
genome or antigenome incorporates a heterologous sequence from
25   HPIV1, HPIV2, HPIV3, BPIV or MPIV to form a chimeric genome or
antigenome.

34. The isolated polynucleotide molecule of claim
33, wherein said chimeric genome or antigenome incorporates
one or more *ts* mutations.

35.   The isolated polynucleotide molecule of claim
33, wherein said chimeric genome or antigenome incorporates
one or more non-*ts* attenuating mutations.

36.   The isolated polynucleotide molecule of claim
33, wherein said chimeric genome or antigenome incorporates
one or more mutations of JS *cp*45.

37.   The isolated polynucleotide molecule of claim
36, wherein said one or more mutations of JS *cp*45 occur in one
or more PIV proteins selected from L, M, N, C, F, or HN or in
a PIV extragenic sequence selected from a 3' leader or N-gene
start sequence.

38.   The isolated polynucleotide molecule of claim
33, wherein said chimeric genome or antigenome incorporates
multiple mutations each specifying a phenotype selected from
attenuation, temperature-sensitivity, cold-adaptation, small
plaque size, or host range restriction.

39.   The isolated polynucleotide molecule of claim
33, wherein said chimeric genome or antigenome incorporates at
least one and up to a full complement of mutations present in
r*cp*45, r*cp*45 3'NCMFHN, r*cp*45 3'NL, r*cp*45 3'N, or r*cp*45 F.

40.   The isolated polynucleotide molecule of claim
33, wherein a mutation specifying a phenotypic alteration
selected from attenuation, temperature-sensitivity, cold-
adaptation, small plaque size, host range restriction, or a
change in an immunogenic epitope of PIV is incorporated in a
chimeric PIV background comprising a genome or antigenome
having one or more PIV3 HN or F glycoprotein genes substituted
by one or more counterpart PIV1 or PIV2 HN and F glycoprotein
genes.

41.   The isolated polynucleotide molecule of claim
33, wherein the heterologous sequence specifies a phenotypic
alteration selected from attenuation, temperature-sensitivity,

cold-adaptation, small plaque size, host range restriction, or a change in an immunogenic epitope of a chimeric PIV.

42.   The isolated polynucleotide molecule of claim 11, which incorporates a cis-acting regulatory sequence of HPIV1, HPIV2, BPIV or MPIV.

43.   The isolated polynucleotide molecule of claim 11, which incorporates a heterologous sequence from RSV.

44.   The isolated polynucleotide molecule of claim 43, wherein the heterologous sequence from RSV is a G or F gene or gene segment.

45.   The isolated polynucleotide molecule of claim 11, which incorporates a heterologous sequence from measles virus.

46.   The isolated polynucleotide molecule of claim 45, wherein the heterologous sequence from measles virus is a HA or F gene or gene segment.

47.   The isolated polynucleotide molecule of claim 11, which incorporates a polynucleotide sequence encoding a non-PIV molecule selected from a cytokine, a T-helper epitope, a restriction site marker, or a protein of a microbial pathogen capable of eliciting a protective immune response in a mammalian host.

48.   A cell or cell-free composition including an expression vector which comprises an isolated polynucleotide molecule encoding a PIV genome or antigenome and an expression vector which comprises one or more isolated polynucleotide molecules that encode(s) N, P and L proteins of PIV, whereby expression of said PIV genome or antigenome and N, P, and L proteins yields an infectious PIV particle.

49.   The cell or cell-free composition of claim 48,
wherein the infectious PIV particle is a virus.

50.   The cell or cell-free composition of claim 48,
wherein the infectious PIV particle is a subviral particle.

5   51.   The cell or cell-free composition of claim 48,
wherein the polynucleotide encoding the PIV genome or
antigenome and the one or more polynucleotides encoding N, P
and L proteins of PIV are incorporated within a single vector.

52.   A method for producing an infectious PIV
10   particle from one or more isolated polynucleotide molecules
encoding said PIV, comprising:
coexpressing in a cell or cell-free system an
expression vector which comprises a polynucleotide molecule
encoding a PIV genome or antigenome and an expression vector
15   which comprises one or more polynucleotide molecules encoding
N, P and L proteins, thereby producing an infectious PIV
particle.

53.   The method of claim 52, wherein the PIV genome
or antigenome and the N, P, and L proteins are expressed by
20   the same expression vector.

54.   The method of claim 52, wherein the N, P, and L
proteins are encoded on two or three different expression
vectors.

55.   The method of claim 52, wherein at least one of
25   the N, P and L proteins is supplied by coinfection with PIV.

56.   The method of claim 52, wherein the
polynucleotide molecule that encodes the PIV genome or
antigenome is cDNA.

57.   The method of claim 52, wherein the infectious
30   PIV particle is a virus.

58. The method of claim 52, wherein the infectious PIV particle is a subviral particle.

59. The method of claim 52, wherein the polynucleotide molecule encoding the PIV genome or antigenome is a human, bovine or murine PIV sequence.

60. The method of claim 52, wherein the polynucleotide molecule encoding the PIV genome or antigenome encodes the sequence of a wild-type PIV strain.

61. The method of claim 52, wherein the polynucleotide molecule encoding the PIV genome or antigenome encodes HPIV3.

62. The method of claim 52, wherein the polynucleotide molecule encoding the PIV genome or antigenome incorporates an attenuating mutation from a biologically derived PIV strain.

63. The method of claim 52, wherein the polynucleotide molecule encoding the PIV genome or antigenome incorporates one or more *ts* mutations.

64. The method of claim 52, wherein the polynucleotide molecule encoding the PIV genome or antigenome incorporates one or more non-*ts* attenuating mutations.

65. The method of claim 52, wherein the polynucleotide molecule encoding the PIV genome or antigenome incorporates at least one mutation of JS *cp*45.

66. The method of claim 65, wherein the polynucleotide molecule encoding the PIV genome or antigenome incorporates multiple mutations of JS *cp*45.

67.   The method of claim 65, wherein the mutation of
JS cp45 specifies at least one amino acid substitution in the
polymerase L protein.

68.   The method of claim 67, wherein the amino acid
substitution in the polymerase L occurs at a position
corresponding to Tyr_{942}, Leu_{992}, or Thr_{1558} of JS cp45.

69.   The method of claim 65, wherein said mutation
of JS cp45 specifies a change in a PIV protein selected from
L, M, N, C, F, or HN or in a PIV extragenic sequence selected
from a 3' leader or N gene start sequence.

70.   The method of claim 52, wherein said
polynucleotide molecule encoding the PIV genome or antigenome
incorporates a mutation that is stabilized by multiple
nucleotide substitutions in a codon which specifies the
mutation.

71.   The method of claim 52, wherein said
polynucleotide molecule encoding said PIV genome or antigenome
incorporates a plurality and up to a full complement of
mutations present in rcp45, rcp45 3'NCMFHN, rcp45 3'NL, rcp45
3'N, or rcp45 F.

72.   The method of claim 69, wherein said
polynucleotide molecule encoding said PIV genome or antigenome
is an antigenomic cDNA selected from rcp45, rcp45 3'NCMFHN,
rcp45 3'NL, rcp45 3'N, rcp45 L, rcp45 F, rcp45 M, rcp45 HN, or
rcp45 C.

73.   The method of claim 52, wherein said
polynucleotide molecule encoding said PIV genome or antigenome
incorporates a heterologous sequence from HPIV1, HPIV2, HPIV3,
BPIV or MPIV to form a chimeric genome or antigenome.

74.   The method of claim 73, wherein the
polynucleotide molecule encoding the PIV genome or antigenome

is a chimera of a HPIV3 sequence and a HPIV1, HPIV2, BPIV or MPIV sequence.

75.   The method of claim 74, wherein a heterologous sequence from HPIV1 or HPIV2 encoding a gene or gene segment
5   of an HN or F glycoprotein is substituted for a corresponding gene or gene segment of HPIV3.

76.   The method of claim 73, wherein said chimeric genome or antigenome incorporates one or more *ts* mutations.

77.   The method of claim 73, wherein said chimeric
10   genome or antigenome incorporates one or more non-*ts* attenuating mutations.

78.   The method of claim 73, wherein said chimeric genome or antigenome incorporates one or more mutations of JS *cp*45.

15   79.   The method of claim 73, wherein said chimeric genome or antigenome incorporates multiple mutations each specifying a phenotype selected from attenuation, temperature-sensitivity, cold-adaptation, small plaque size, or host range restriction.

20   80.   The method of claim 73, wherein a mutation specifying a phenotypic alteration selected from attenuation, temperature-sensitivity, cold-adaptation, small plaque size, host range restriction, or a change in an immunogenic epitope of PIV is incorporated in a chimeric PIV background comprising
25   a genome or antigenome having one or more PIV3 HN or F glycoprotein genes substituted by one or more counterpart PIV1 or PIV2 HN and F glycoprotein genes.

81.   The method of claim 80, wherein one or more mutations of JS *cp*45 are incorporated in a chimeric background
30   comprising a genome or antigenome having both PIV3 HN and F

glycoprotein genes substituted by counterpart PIV1 or PIV2 HN
and F glycoprotein genes.

82.   The method of claim 81, wherein said one or
more mutations of JS cp45 occur in one or more PIV proteins
selected from L, M, N, C, F, or HN or in a PIV extragenic
sequence selected from a 3' leader or N gene start sequence.

83.   The method of claim 73, wherein the
heterologous sequence specifies a phenotypic alteration
selected from attenuation, temperature-sensitivity, cold-
adaptation, small plaque size, host range restriction, or a
change in an immunogenic epitope of a chimeric PIV.

84.   The method of claim 52, wherein said
polynucleotide molecule encoding said PIV genome or antigenome
incorporates a heterologous sequence from RSV.

85.   The method of claim 84, wherein the
heterologous sequence from RSV is a G or F gene or gene
segment.

86.   The method of claim 52, wherein said
polynucleotide molecule encoding said PIV genome or antigenome
incorporates a heterologous sequence from measles virus.

87.   The method of claim 86, wherein the
heterologous sequence from measles virus is a HA or F gene or
gene segment.

88.   The method of claim 52, wherein the
polynucleotide molecule encoding the PIV genome or antigenome
is selected from:
        i)   p218(131) (SEQ ID NO: 1);
        ii)   p3/7(131) (SEQ ID NO: 14);
        iii)   p3/7(131)2G (SEQ ID NO: 15); or
        iv)   the polynucleotide molecule of i), ii) or iii)
modified by introduction of a heterologous PIV sequence

selected from a HPIV1 sequence, a HPIV2 sequence, a BPIV
sequence or a MPIV sequence or by a nucleotide insertion,
rearrangement, deletion or substitution specifying a
phenotypic alteration selected from attenuation, temperature-
5   sensitivity, cold-adaptation, small plaque size, host range
restriction, or a change in an immunogenic epitope of PIV.

         89.   The method of claim 52, wherein the
polynucleotide molecule encoding the PIV genome or antigenome
is selected from:
10   i)   p218(131) (SEQ ID NO: 1);
              ii)   p3/7(131) (SEQ ID NO: 14);
              iii) p3/7(131)2G (SEQ ID NO: 15); or
              iv)   the polynucleotide molecule of i), ii) or iii)
modified by introduction of a heterologous PIV sequence
15   selected from a HPIV1 sequence, a HPIV2 sequence, a BPIV
sequence or a MPIV sequence and by a nucleotide insertion,
rearrangement, deletion or substitution different from said
introduction of said heterologous PIV sequence specifying a
phenotypic alteration selected from attenuation, temperature-
20   sensitivity, cold-adaptation, small plaque size, host range
restriction, or a change in an immunogenic epitope of PIV.

         90.   The method of claim 52, wherein the
polynucleotide molecule encoding the PIV genome or antigenome
is modified to encode a non-PIV molecule selected from a
25   cytokine, a T-helper epitope, a restriction site marker, or a
protein of a microbial pathogen capable of eliciting a
protective immune response in a mammalian host.

         91.   An isolated infectious PIV particle which
comprises a recombinant PIV genome or antigenome, a N protein,
30   a P protein, and a L protein.

         92.   The isolated infectious PIV particle of claim
91, which is a subviral particle.

93.   The isolated infectious PIV particle of claim
91, which is a virus.

94.   The isolated infectious PIV particle of claim
91, wherein the recombinant PIV genome or antigenome

5   incorporates a heterologous sequence from RSV or measles
virus.

95.   The isolated infectious PIV particle of claim
91, wherein the recombinant PIV genome or antigenome is a
cDNA.

10   96.   The isolated infectious PIV particle of claim
91, which is a human PIV.

97.   The isolated infectious PIV particle of claim
91, wherein the recombinant PIV genome or antigenome is a
chimera of heterologous PIV sequences selected from HPIV1,

15   HPIV2, HPIV3, BPIV, or MPIV sequences.

98.   The isolated infectious PIV particle of claim
91, wherein the recombinant PIV genome or antigenome is
selected from:
            i)   p218(131) (SEQ ID NO: 1);

20   ii)   p3/7(131) (SEQ ID NO: 14);
            iii) p3/7(131)2G (SEQ ID NO: 15); or
            iv)   the genome or antigenome of i), ii) or iii)
modified by introduction of a heterologous PIV sequence
selected from a HPIV1 sequence, a HPIV2 sequence, a BPIV

25   sequence or a MPIV sequence or by a nucleotide insertion,
rearrangement, deletion or substitution specifying a
phenotypic alteration selected from attenuation, temperature-
sensitivity, cold-adaptation, small plaque size, host range
restriction, or a change in an immunogenic epitope of PIV.

30   99.   The isolated infectious PIV particle of claim
91, wherein the recombinant PIV genome or antigenome is
selected from:

    i)   p218(131) (SEQ ID NO: 1);

    ii)   p3/7(131) (SEQ ID NO: 14);

    iii) p3/7(131)2G (SEQ ID NO: 15); or

    iv)   the genome or antigenome of i), ii) or iii)

5   modified by introduction of a heterologous PIV sequence
    selected from a HPIV1 sequence, a HPIV2 sequence, a BPIV
    sequence or a MPIV sequence and by a nucleotide insertion,
    rearrangement, deletion or substitution different from said
    introduction of said heterologous PIV sequence specifying a
10 phenotypic alteration selected from attenuation, temperature-
    sensitivity, cold-adaptation, small plaque size, host range
    restriction, or a change in an immunogenic epitope of PIV.

        100. The isolated infectious PIV particle of claim
    91, wherein the counterpart gene or gene segment is a gene or
15 gene segment of the HN or F glycoprotein gene of HPIV1 or
    HPIV2.

        101. The isolated infectious PIV particle of claim
    91, wherein the recombinant PIV genome or antigenome
    incorporates a heterologous sequence from RSV or measles
20 virus.

        102. The isolated infectious PIV particle of claim
    91, wherein the recombinant PIV genome or antigenome is
    modified by a nucleotide insertion, rearrangement, deletion or
    substitution encoding a phenotypic alteration selected from
25 attenuation, temperature-sensitivity, cold-adaptation, small
    plaque size, host range restriction, or a change in an
    immunogenic epitope of PIV.

        103. The isolated infectious PIV particle of claim
    91, wherein the recombinant PIV genome or antigenome
30 incorporates multiple ts mutations.

        104. The isolated infectious PIV particle of claim
    91, wherein the recombinant PIV genome or antigenome
    incorporates multiple non-ts attenuating mutations.

105. The isolated infectious PIV particle of claim 91, wherein the recombinant PIV genome or antigenome incorporates at least one mutation of JS *cp*45.

106. The isolated infectious PIV particle of claim 105, wherein the mutation of JS *cp*45 specifies an amino acid substitution in the polymerase L protein.

107. The isolated infectious PIV particle of claim 97, wherein said chimeric genome or antigenome incorporates one or more *ts* mutations.

108. The isolated infectious PIV particle of claim 97, wherein said chimeric genome or antigenome incorporates one or more non-*ts* attenuating mutations.

109. The isolated infectious PIV particle of claim 97, wherein said chimeric genome or antigenome incorporates one or more mutations of JS *cp*45.

110. The isolated infectious PIV particle of claim 129, wherein said chimeric genome or antigenome incorporates multiple mutations each specifying a phenotype selected from attenuation, temperature-sensitivity, cold-adaptation, small plaque size, or host range restriction.

111. The isolated infectious PIV particle of claim 109, wherein said chimeric genome or antigenome incorporates at least one and up to a full complement of mutations present in r*cp*45, r*cp*45 3'NCMFHN, r*cp*45 3'NL, r*cp*45 3'N, or r*cp*45 F.

112. The isolated infectious PIV particle of claim 91, wherein a mutation specifying a phenotypic alteration selected from attenuation, temperature-sensitivity, cold-adaptation, small plaque size, host range restriction, or a change in an immunogenic epitope of PIV is incorporated in a chimeric PIV background comprising a genome or antigenome having one or more PIV3 HN or F glycoprotein genes or gene

segments substituted by one or more counterpart PIV1 or PIV2
HN and F glycoprotein genes or gene segments.

113. The isolated infectious PIV particle of claim
112, wherein one or more mutations of JS cp45 are incorporated
in said chimeric background.

114. The isolated infectious PIV particle of claim
of claim 113, wherein said one or more mutations of JS cp45
occur in one or more PIV proteins selected from L, M, N, C, F,
or HN or in a PIV extragenic sequence selected from a 3'
leader or N gene start sequence.

115. The isolated infectious PIV particle of claim
91, wherein the recombinant PIV genome or antigenome is
modified to encode a non-PIV molecule selected from a
cytokine, a T-helper epitope, a restriction site marker, or a
protein of a microbial pathogen capable of eliciting a
protective immune response in a mammalian host.

116. The isolated infectious PIV particle of claim
91, further comprising an RSV antigen or epitope which elicits
protective immunity to RSV in an immunized host.

117. The isolated infectious PIV particle of claim
91, which is selected from r942, r992, r1558, r942/992,
r992/1558, r942/1558, or r942/992/1558, rcp45 3'N, rcp45 C,
rcp45 M, rcp45 F, rcp45 HN, rcp45L, rcp45 3'NL, rcp45
3'NCMFHN, and rcp45.

118. An immunogenic composition comprising an
immunogenically effective amount of an infectious PIV particle
in a pharmaceutically acceptable carrier, said PIV particle
comprising a recombinant PIV genome or antigenome, a N
protein, a P protein, and a L protein.

119. The immunogenic composition of claim 118,
wherein said infectious PIV particle is a subviral particle.

120. The immunogenic composition of claim 118, wherein said infectious PIV particle is a virus.

121. The immunogenic composition of claim 118, wherein the recombinant PIV genome or antigenome incorporates a heterologous sequence from RSV or measles virus.

122. The immunogenic composition of claim 118, wherein the recombinant PIV genome or antigenome is a chimera of heterologous PIV sequences selected from HPIV1, HPIV2, HPIV3, BPIV, or MPIV sequences to form an infectious, chimeric PIV particle.

123. The immunogenic composition of claim 118, wherein the recombinant PIV genome or antigenome encodes a human PIV in which a gene or gene segment is replaced with a counterpart gene or gene segment from a heterologous PIV.

124. The immunogenic composition of claim 123, wherein one or both HN and F glycoprotein genes of HPIV1 are substituted for HN and F glycoprotein genes of HPIV3 to form said infectious, chimeric PIV particle.

125. The immunogenic composition of claim 123, wherein the recombinant PIV genome or antigenome of said infectious, chimeric PIV particle is modified by a nucleotide insertion, rearrangement, deletion or substitution encoding a phenotypic alteration selected from attenuation, temperature-sensitivity, cold-adaptation, small plaque size, host range restriction, or a change in an immunogenic epitope of PIV.

126. The immunogenic composition of claim 125, wherein said recombinant PIV genome or antigenome incorporates multiple mutations selected from *ts* and non-*ts* attenuating mutations to form an attenuated, infectious, chimeric PIV particle.

127. The immunogenic composition of claim 118, wherein the recombinant PIV genome or antigenome incorporates a mutation of JS *cp*45.

128. The immunogenic composition of claim 118, wherein the recombinant PIV genome or antigenome incorporates multiple mutations of JS *cp*45.

1/18

FIG. 1
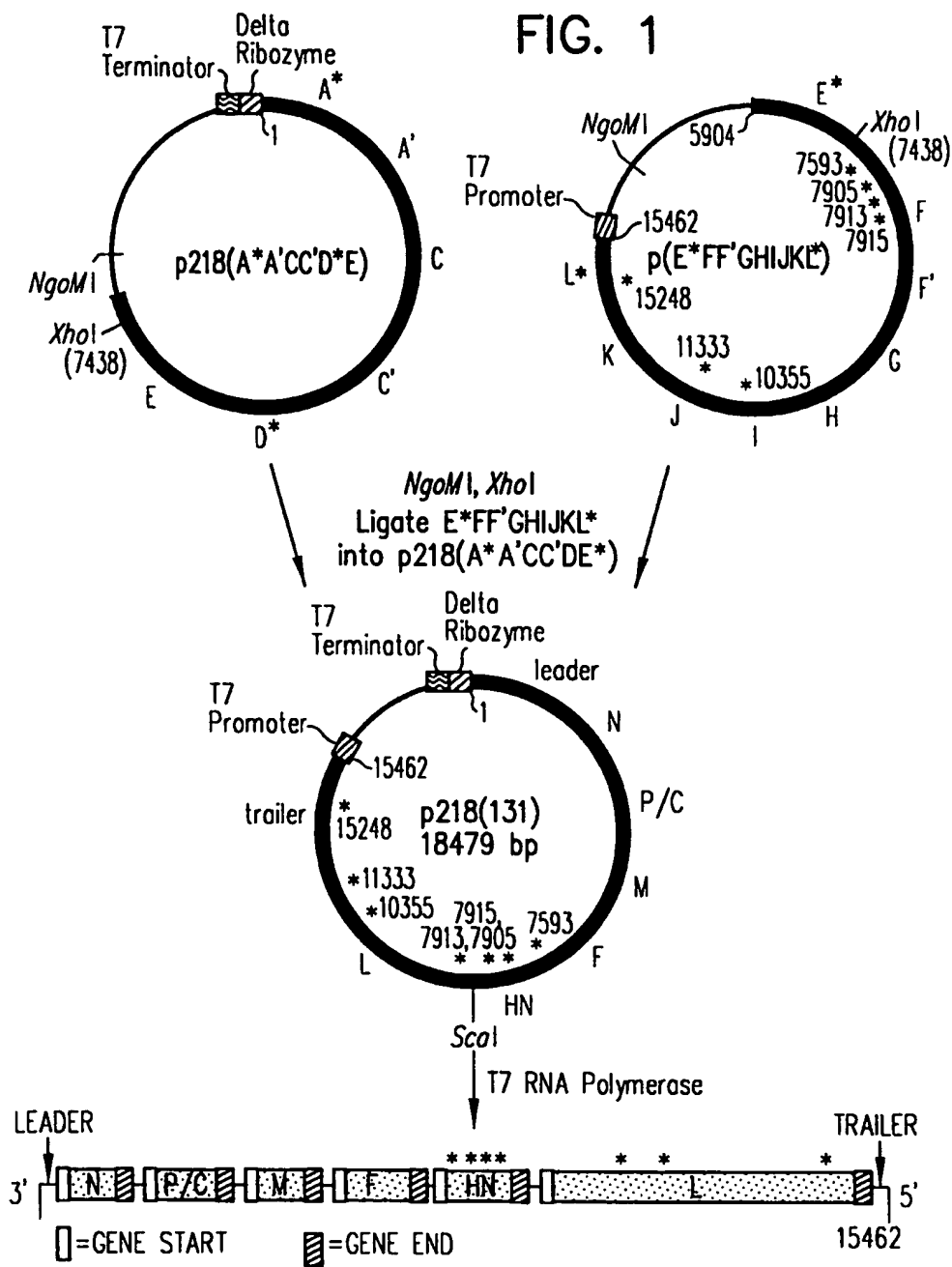
FIG. 2

1) Introduced and Incidental Mutations in HN gene of JS cDNA [p218(131)]

```
                                  Hgal                    PRO370
                 THR263         CAA GCG TCT CAT   AGT CCA TGG TTT
JSwt parent    AAT ACA GAT
                                           ↓                ↓   ↓
JScDNA    7589 AAT A□A GAT   7898 CAA GC□ TCT CAT   AGT AC□ TGG TTT
               ILE263              ScaI        ScaI THR370
```

2) Incidental mutations in the L gene of JScDNA [p218(131)]

```
                                  ASN618                 ILE2221
                 THR595         AAG AAC ATT    TTG ATT GGT
JSwt parent    GCT ACA CAA
                      ↓              ↓              ↓
JScDNA   10350 GCT AC□ CAA  11328 AAG AA□ ATT   15243 TTG AT□ GGT
               THR595              ASN618               ILE2221
```


FIG. 15A

3/18
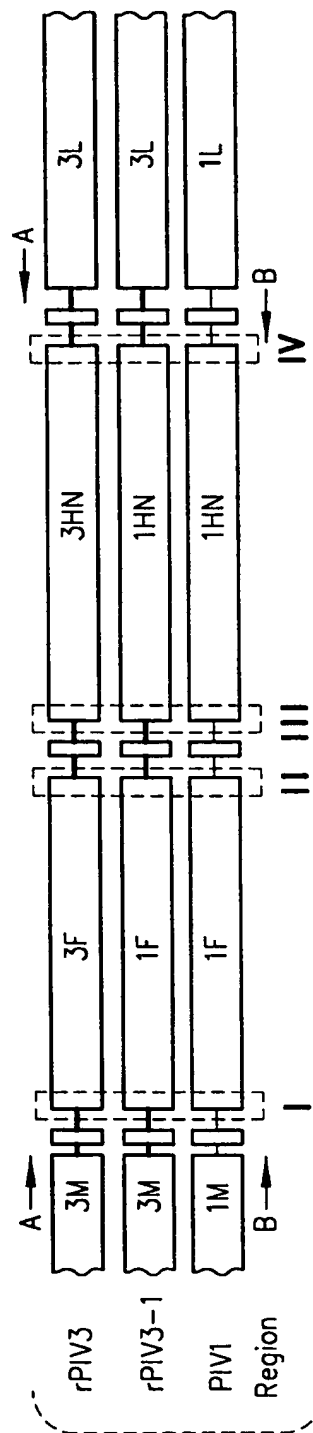
FIG. 3
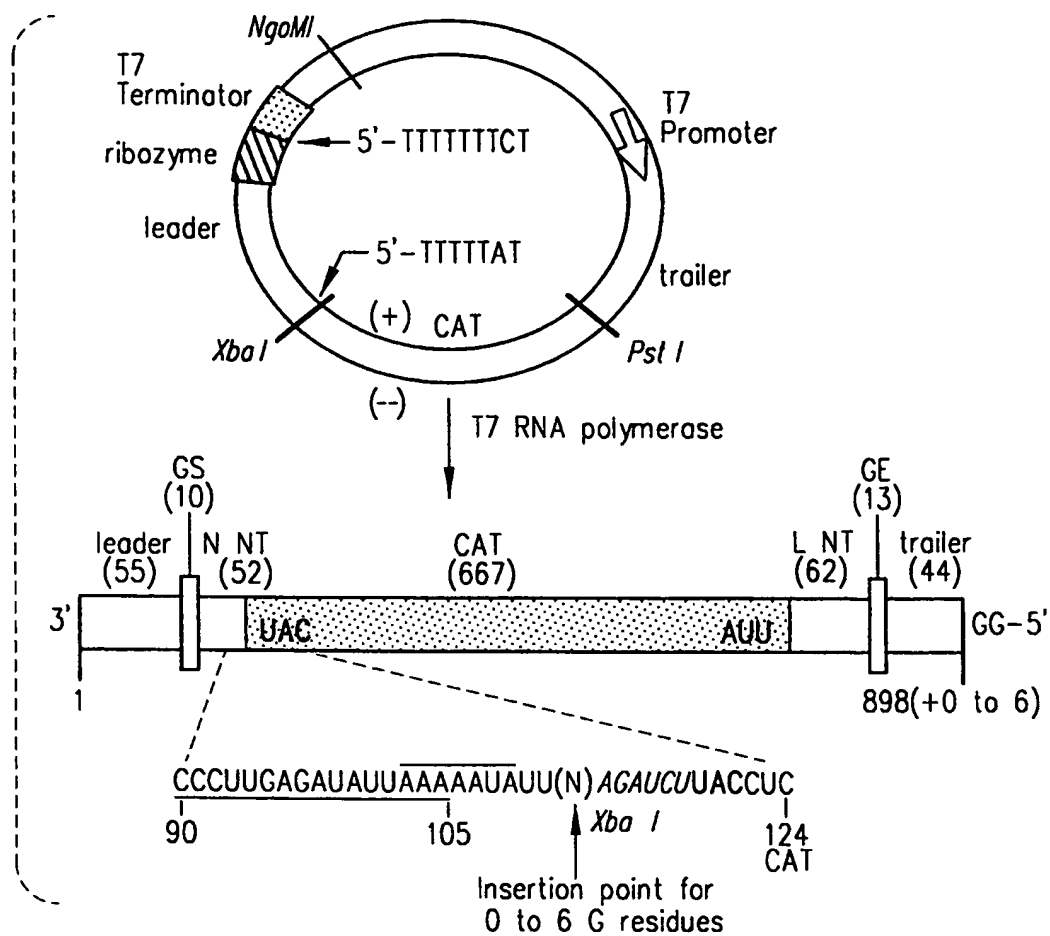
FIG. 4
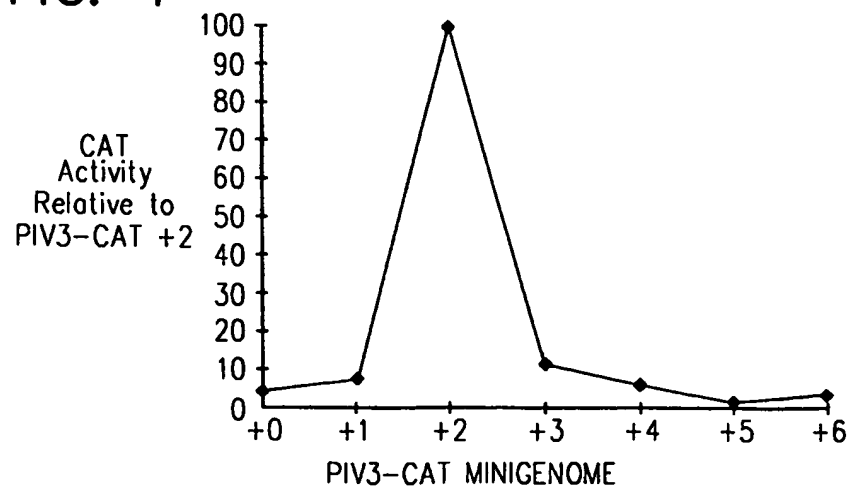
FIG. 5A
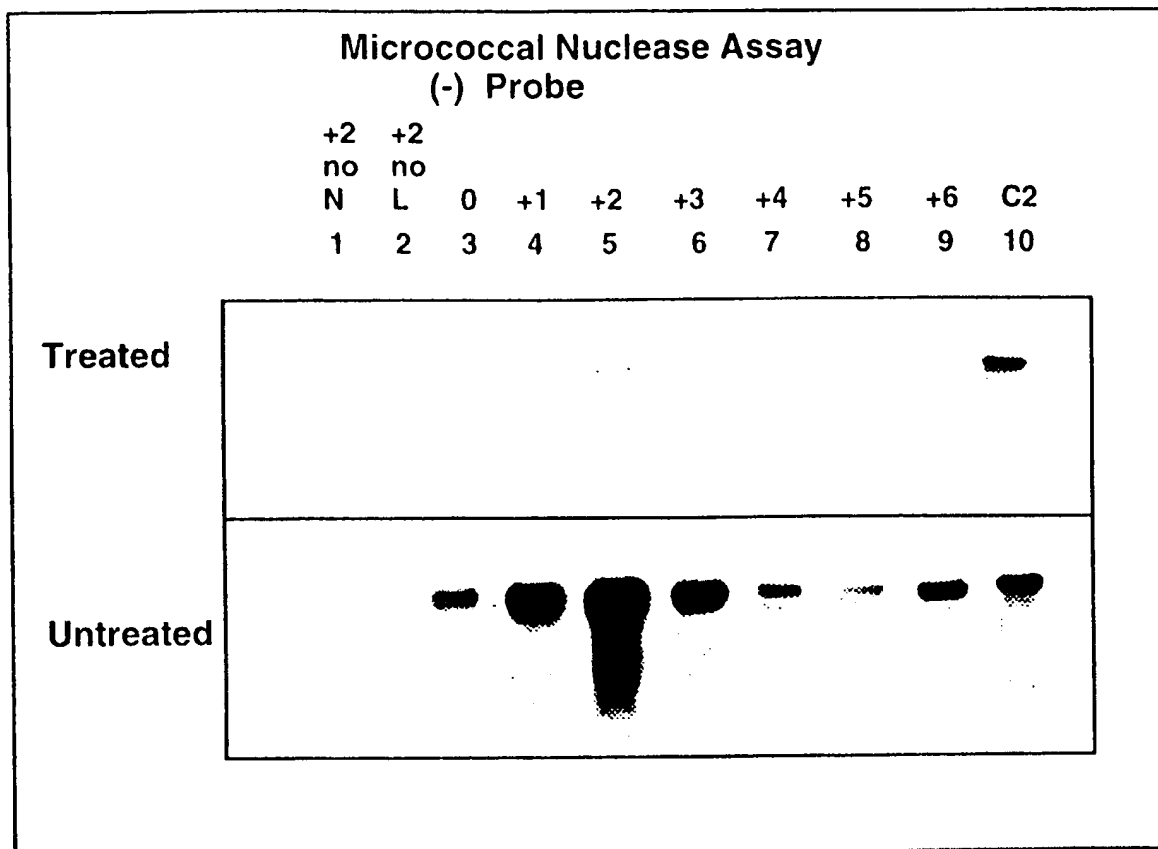
FIG. 5B
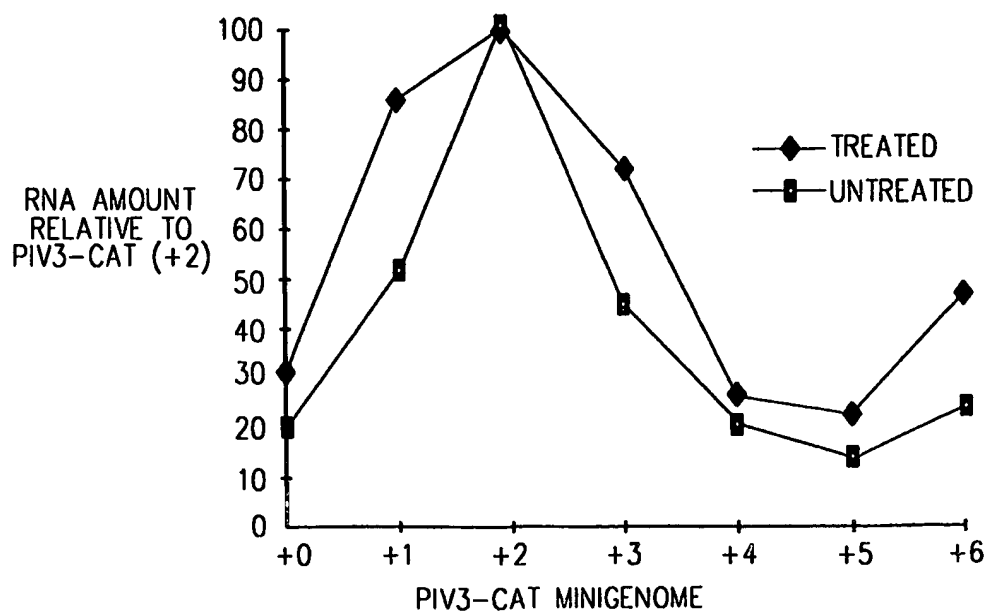
5/18

FIG. 6A
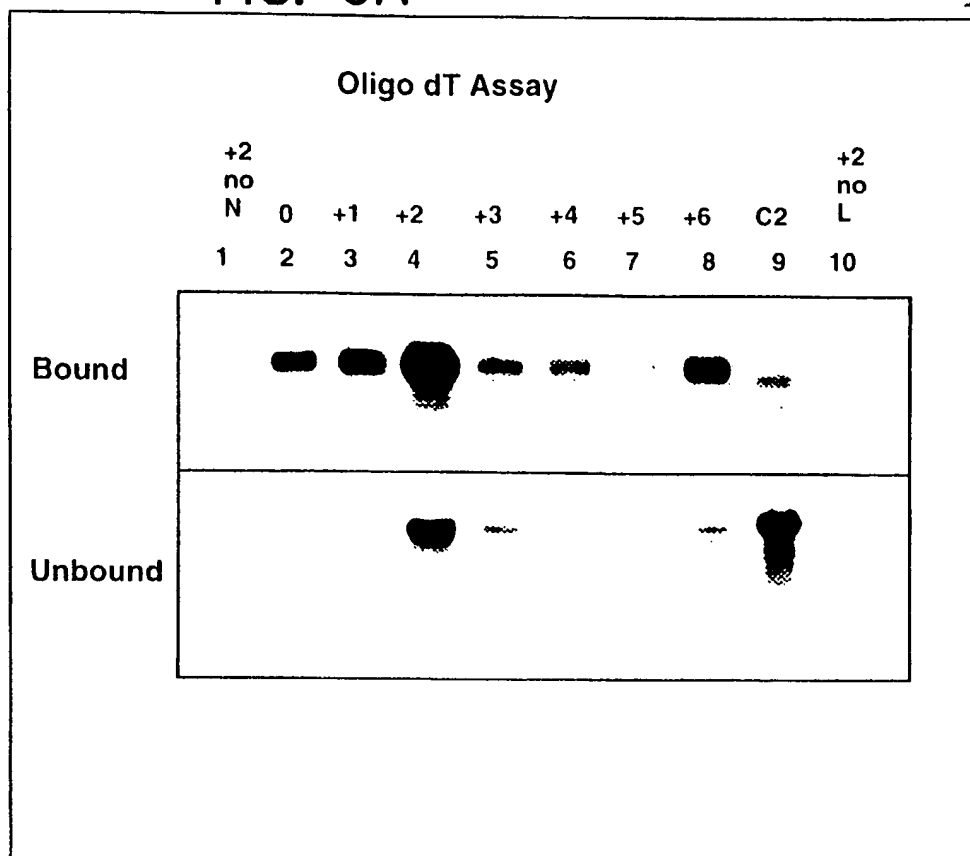
FIG. 6B
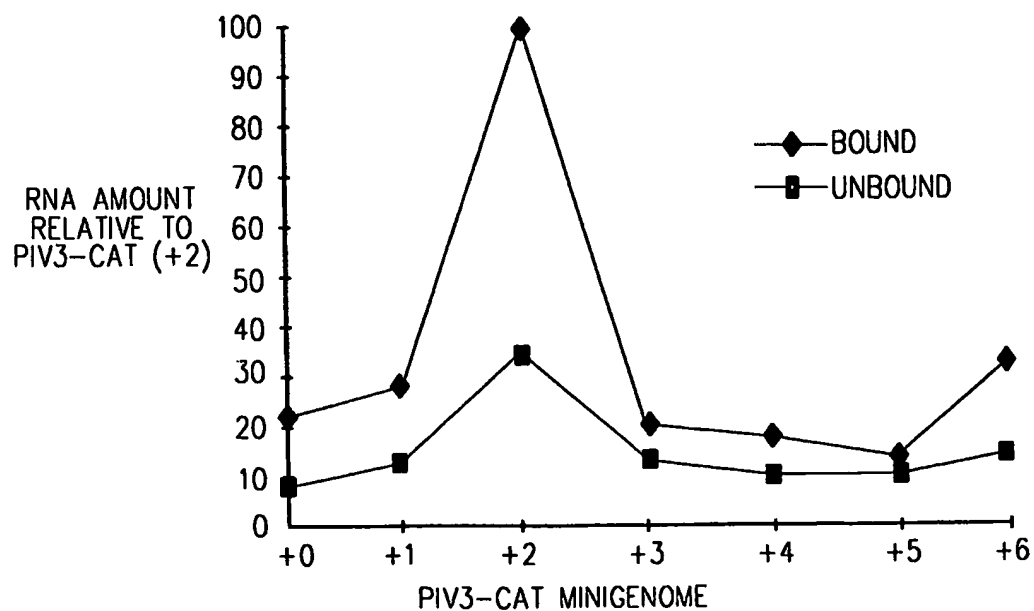
6/18

FIG. 7A
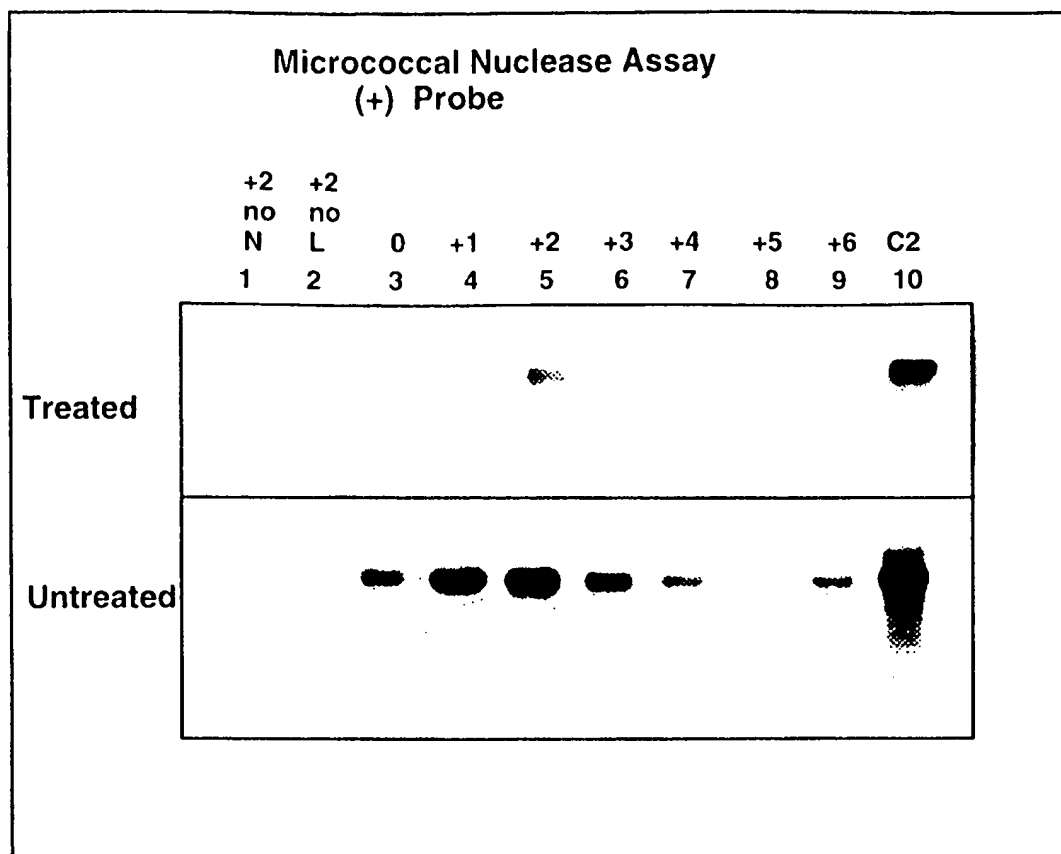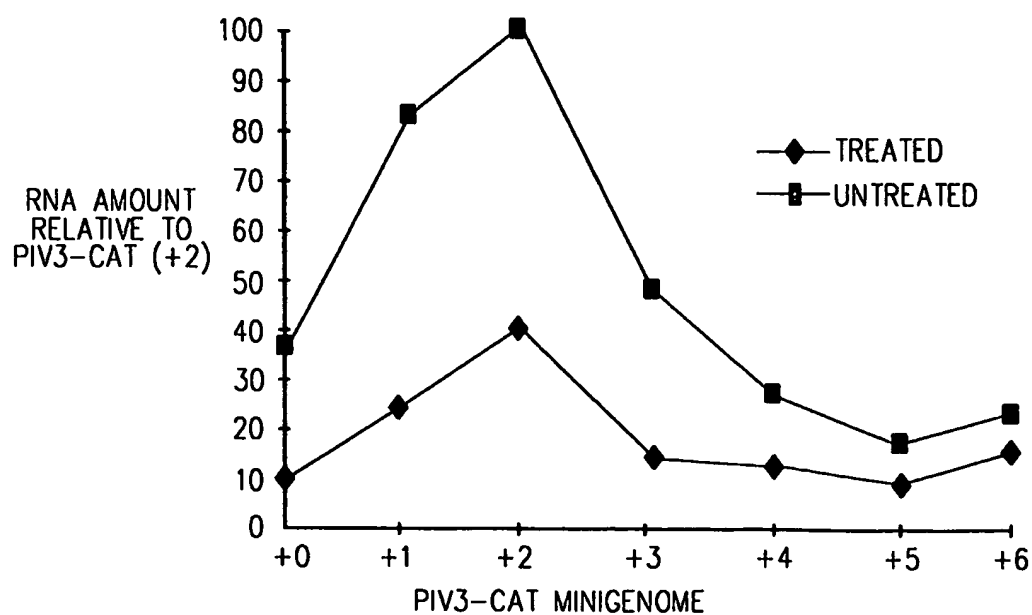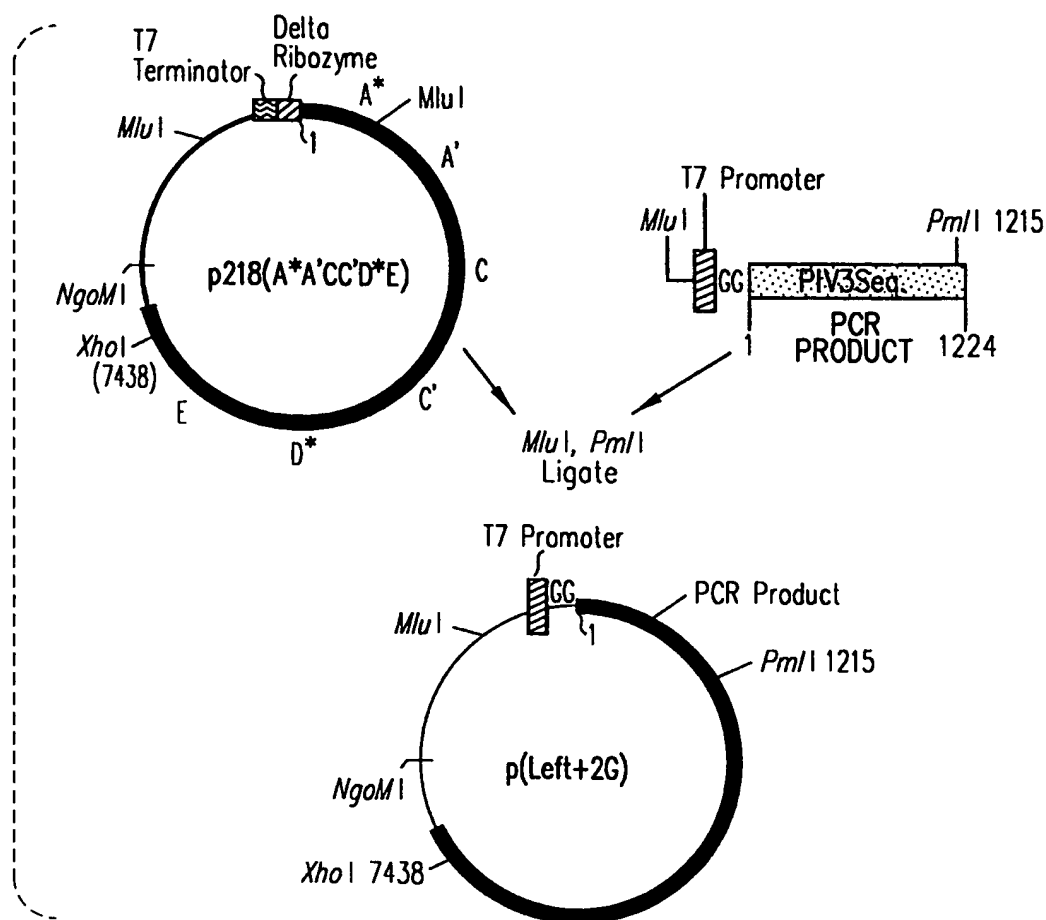
Micrococcal Nuclease Assay
(+) Probe

FIG. 7B

7/18

FIG. 8

FIG. 12
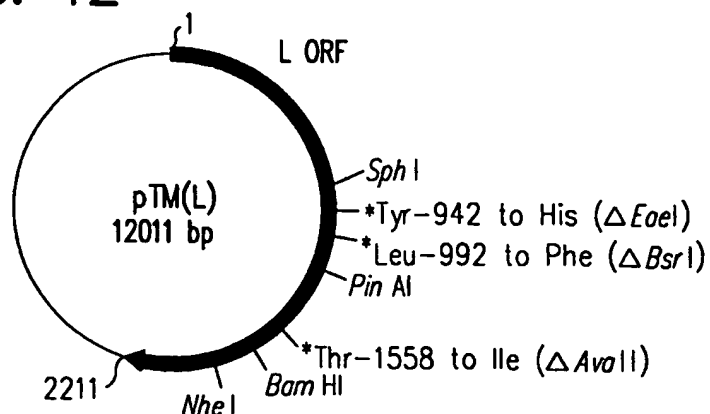
FIG. 9
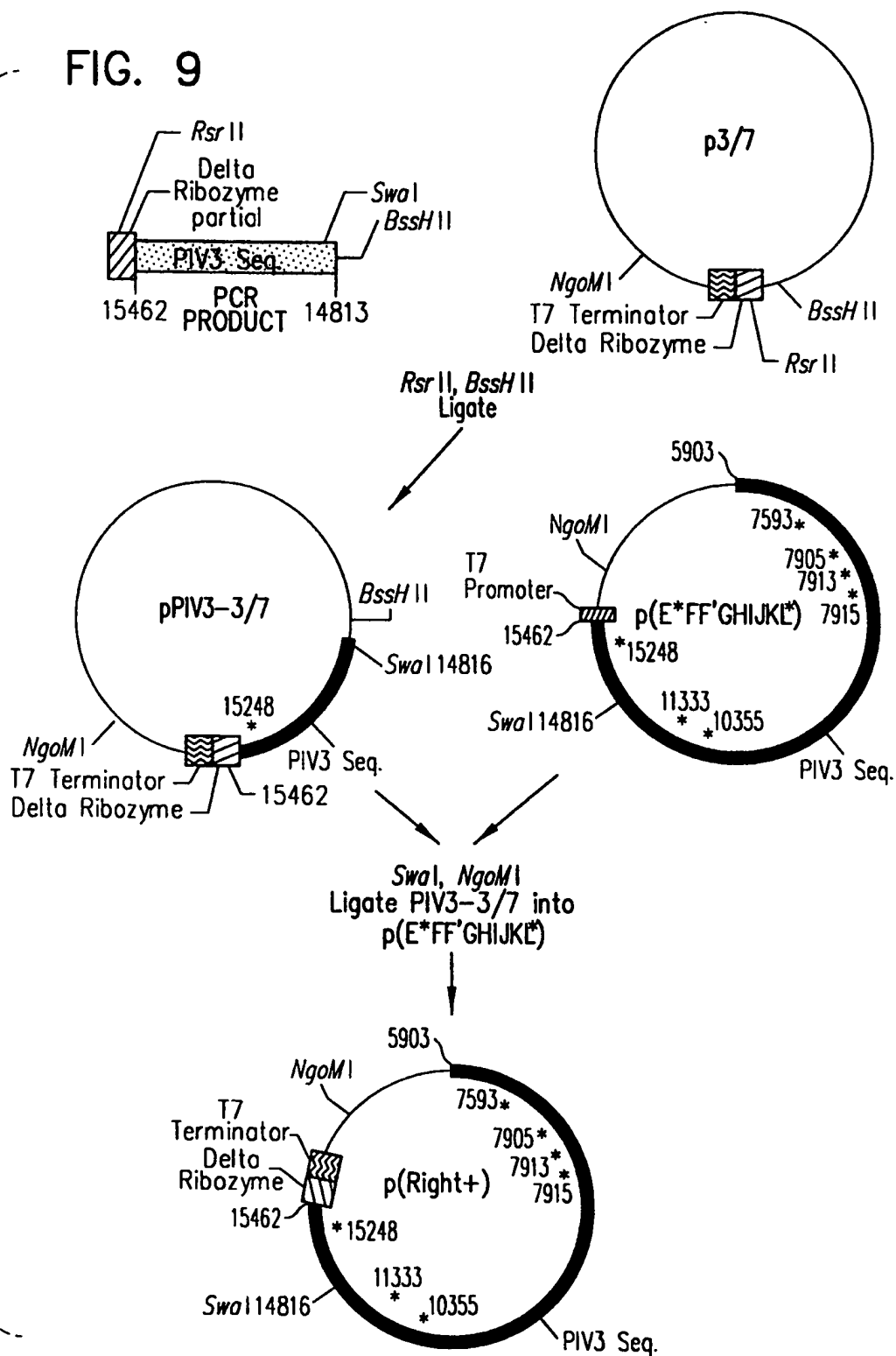
FIG. 10
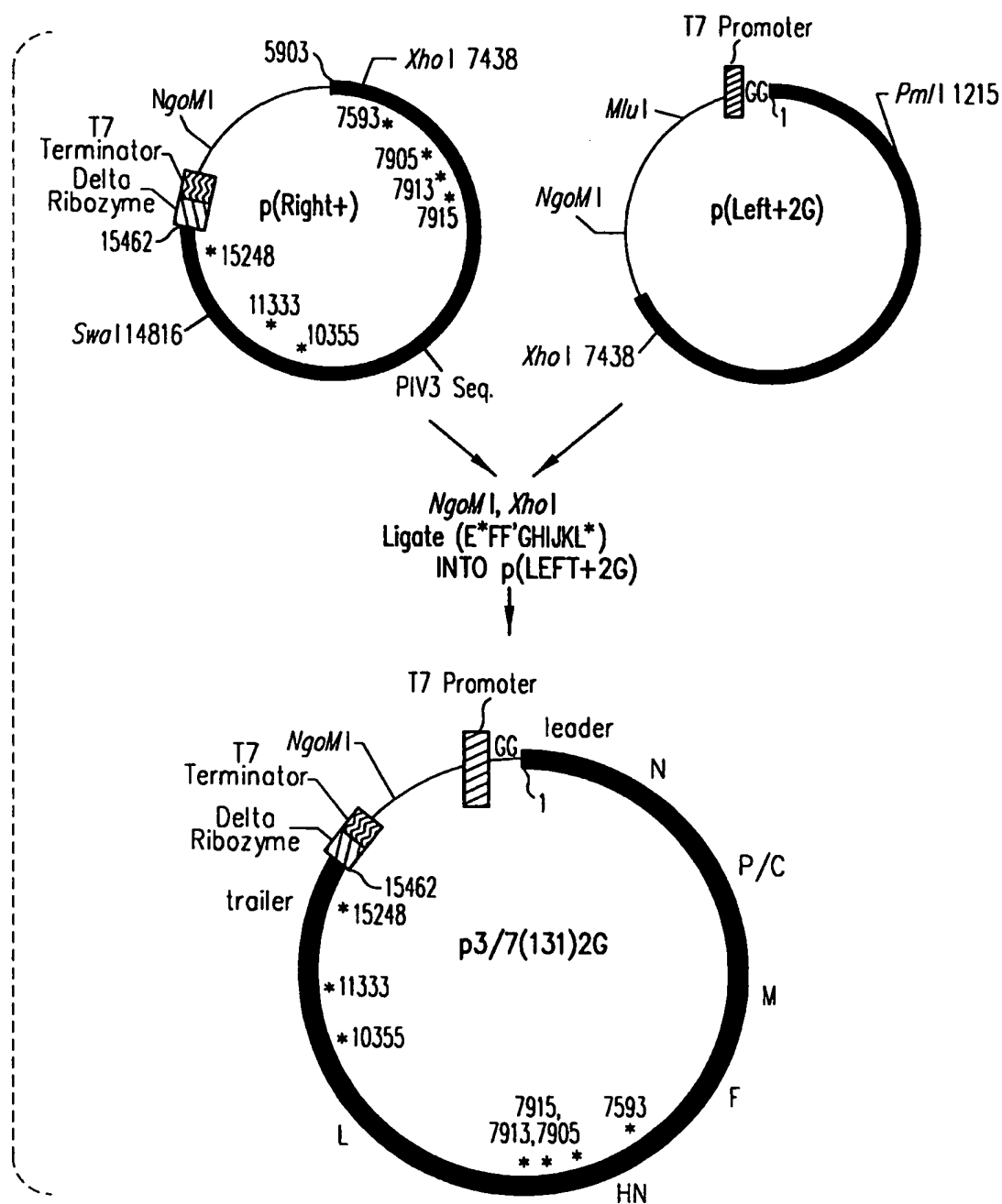
FIG. 11
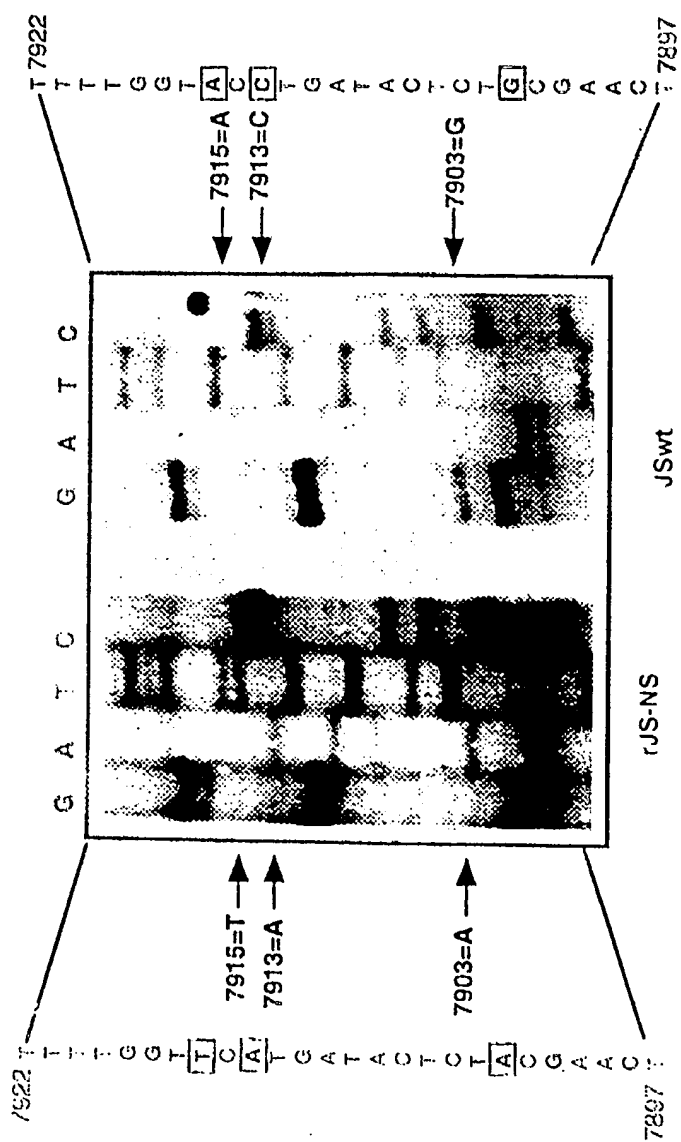
FIG. 13
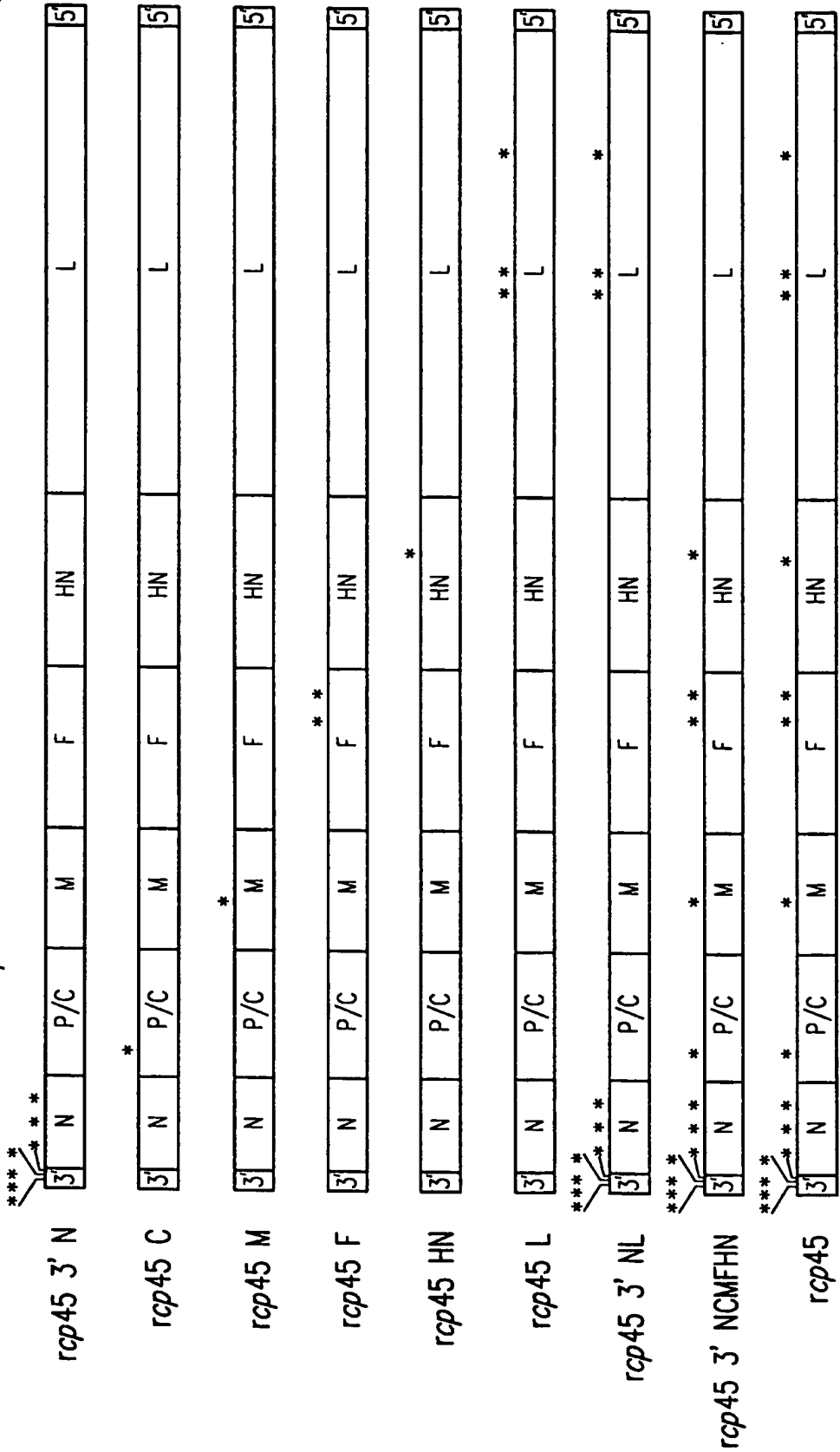
Figure 1: Schematic representation of recombinant PIV3 viruses bearing mutations (∗) in the 3' leader, N gene start, N, C, M, F, HN and/or L proteins.

FIG. 14A

FIG. 14B

14/18

FIG. 15B

FIG. 16
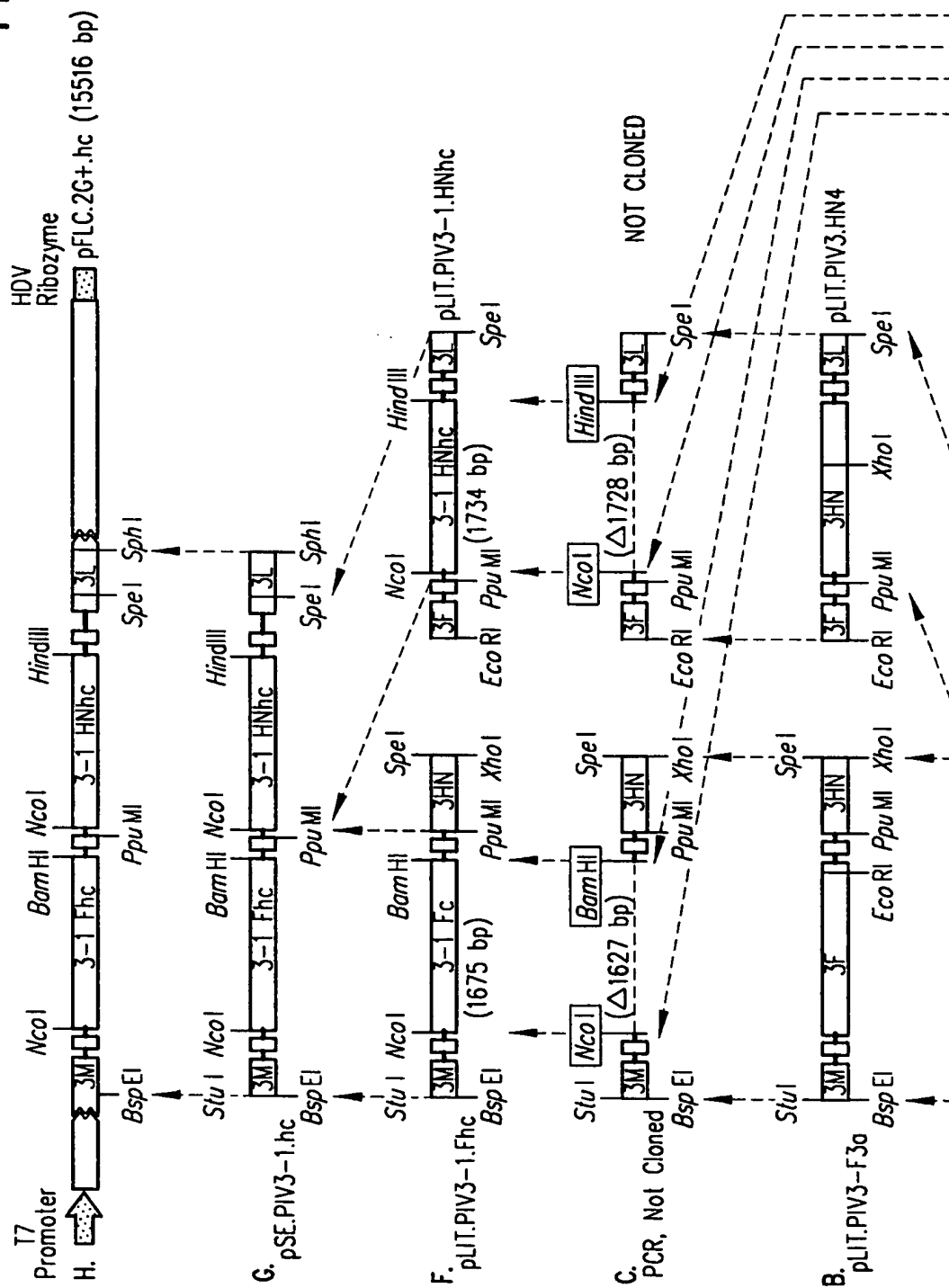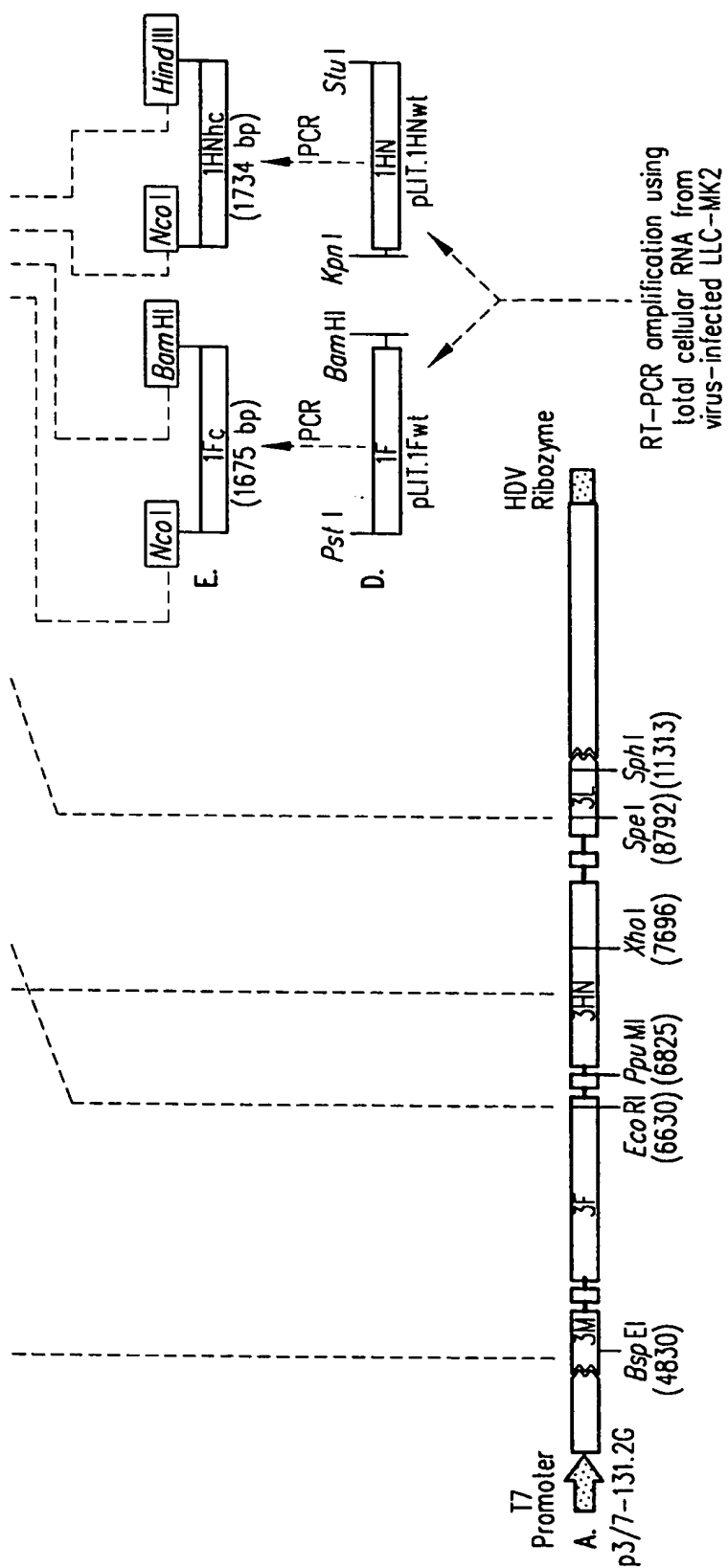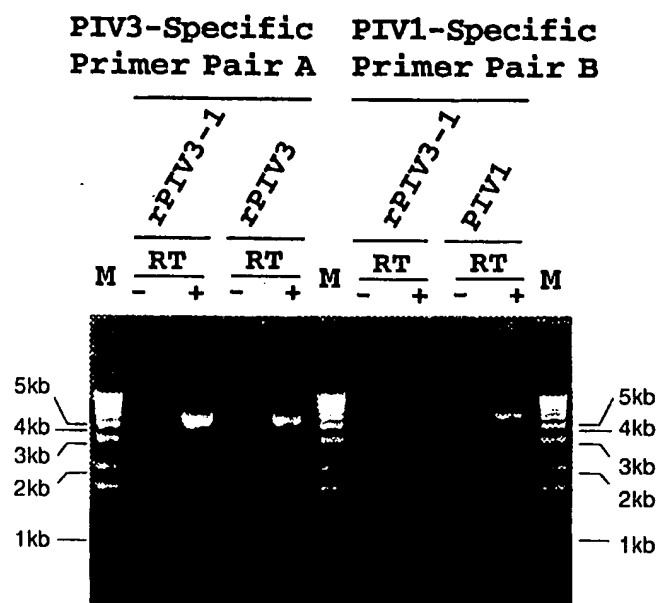
REGION I

```
                                                              ┌─▶ F ORF                           5107
rPIV3  5039  AAGAGACCGGCCAACACAACAAGCACTGAACACATGCCAACTTCAATACTGCTAATTATTACAACCATG
             ||||||||||||||||||||||||||||  ||  |||  ||  |||||||||||||||||
rPIV3-1 5039 AAGAGACCGGCCAACACAACAAGCACTGAAC┌──┐CCATGGAAAAATCAGAGATCCTCTCTTAGTATACTCA  5107
             ||  ||                        |TT|  ||||||||||||||||||||||||||||||||
PIV1         AAATTCATAATAAACACCAAGATAGAGACAAAAATGCAAAAATCAGAGATCCTCTTCTTAGTATACTCA
                                           └──┘
                              ┌── F ORF ──┐
                                 Gln to Glu
```

REGION II

```
rPIV3  6659  AATGACAAGCCATATGTACTAACAAACAAATAACACATATCTACAGATCATTAGATATTAAAATTATAAAA  6727
             |||||||||||||  |||||||||||||||||||||||| |||||||||||||||||||||||||||||
rPIV3-1 6707 AGAAATCCCTACATGGGTAACAACTCCAATT┌──────┐AGGATCCCTACAGATCATTAGATATTAAAATTATAAAA  6775
             |||||||||||||||||||||||||||||||                    ||||||||||||||||||||||||
PIV1         AGAAATCCCTACATGGGTAACAACTCCAATTAAAAAAAATCAGATCAAGTACATTGTAGCATACATACAAC
                                            └──────┘
                                                   ┌─▶ HN ORF
```

REGION III

```
rPIV3  6773  AGGAAGGACCCAATAGACACCAATCCAAATTCGAGATGCAATACTGGAAGCATCAATCACGGAAAGGAT  6841
             |||||||||||||||||||||||||||||||||||||||  |                 |||||||
rPIV3-1 6821 AGGAAGGACCCAATAGACACCAATCCAAATI┌────┐CCATGGCTGAAAAAGGGAAAAACAAATAGTTCATATTGG  6877
             ||||||||||||||||||||||||||||||                     ||||||||||||||||||
PIV1         GAGCTCTGGTACCAGCATCCGATTATACAAACGATGGCTGAAAACGAAGGGAAAAACAAATAGTTCATATTGG
                                            └────┘
                                                   ┌── HN ORF ──┐
```

REGION IV

```
rPIV3  8486  ATGTTGTTCAAAACAGAGATTCCAAAAAGCTGCAGTTAATCATAATTAACCATAATATGCATCAATCTA  8554
             |||||||||||||||||||||||||||||||||  ||||  |     ||||||||||||||||||||||
rPIV3-1 8540 AAGACAAGTATCCCTAAAATATGTAAAATCACATCTT┌──┐AGCTTAATTAACCATAATATGCATCAATCTA  8608
             |||||||||||||||||  ||  |   |||||   |   |  ||||||||||||||||||||||||||||
PIV1         AAGACAAGTATCCCTAAAATATGTAAAATCACATCTTGAGCAGATCAAGGCCTTAAGTGTGTAGTCGTATT
                                            └──┘
```
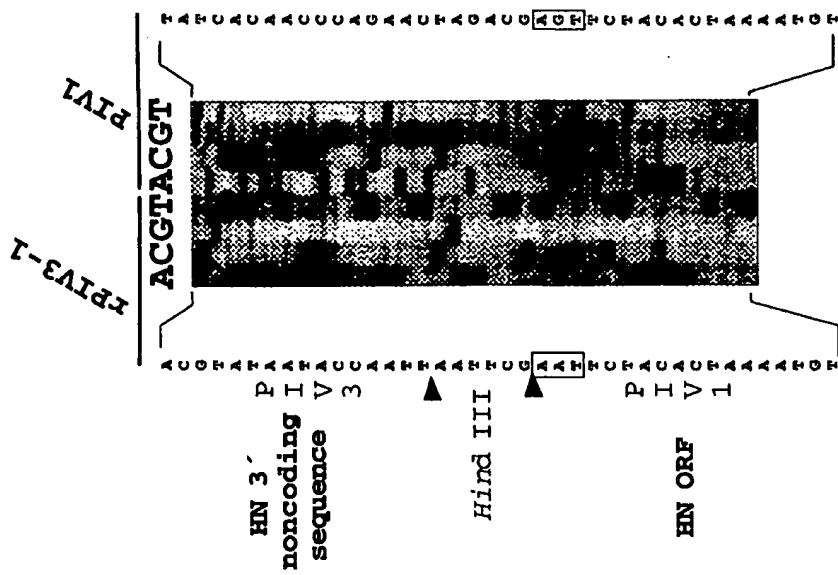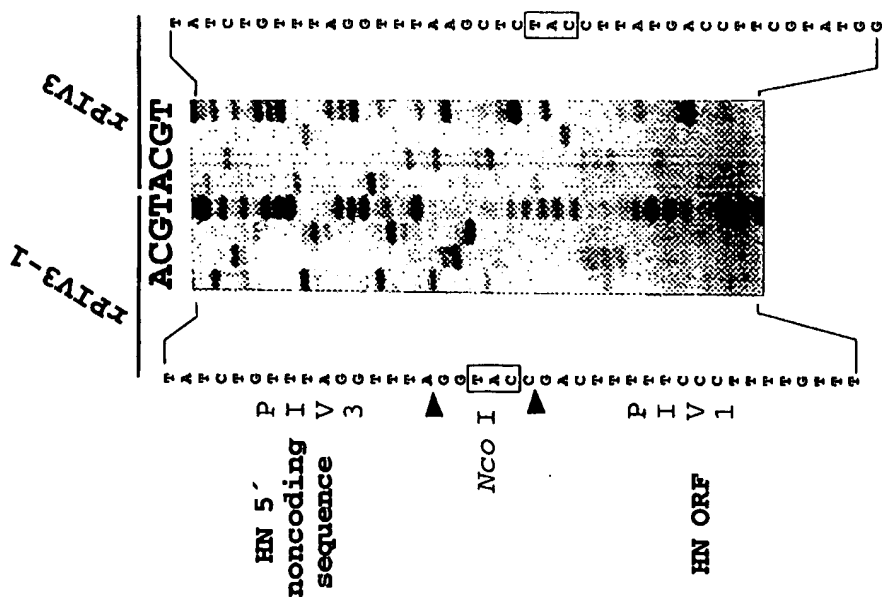
-16/18-

FIG. 17B

FIG. 17A
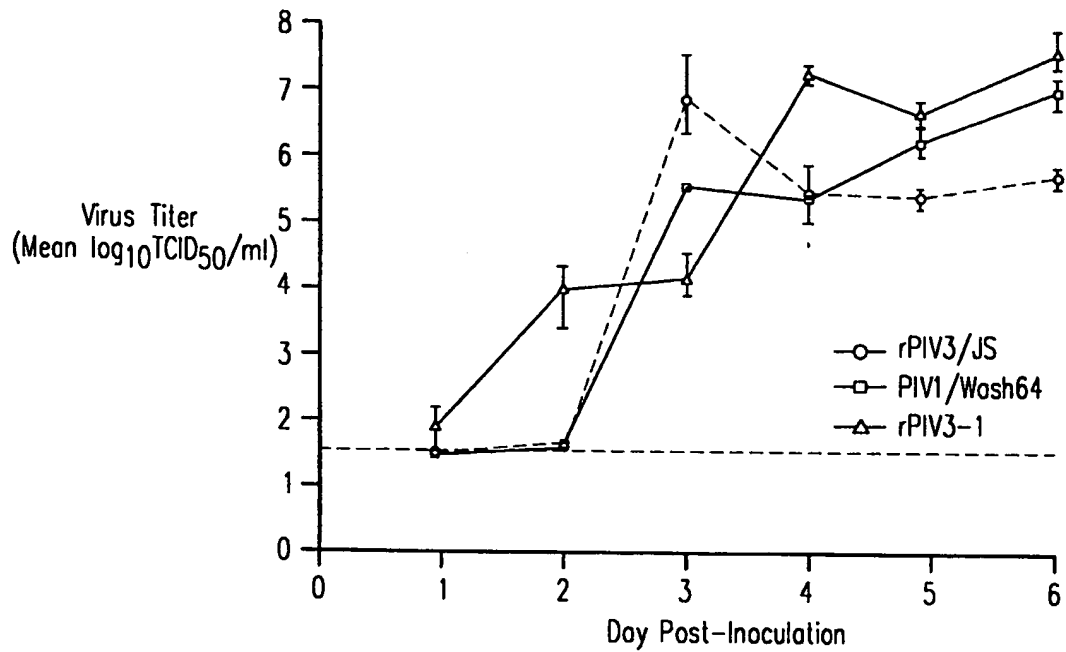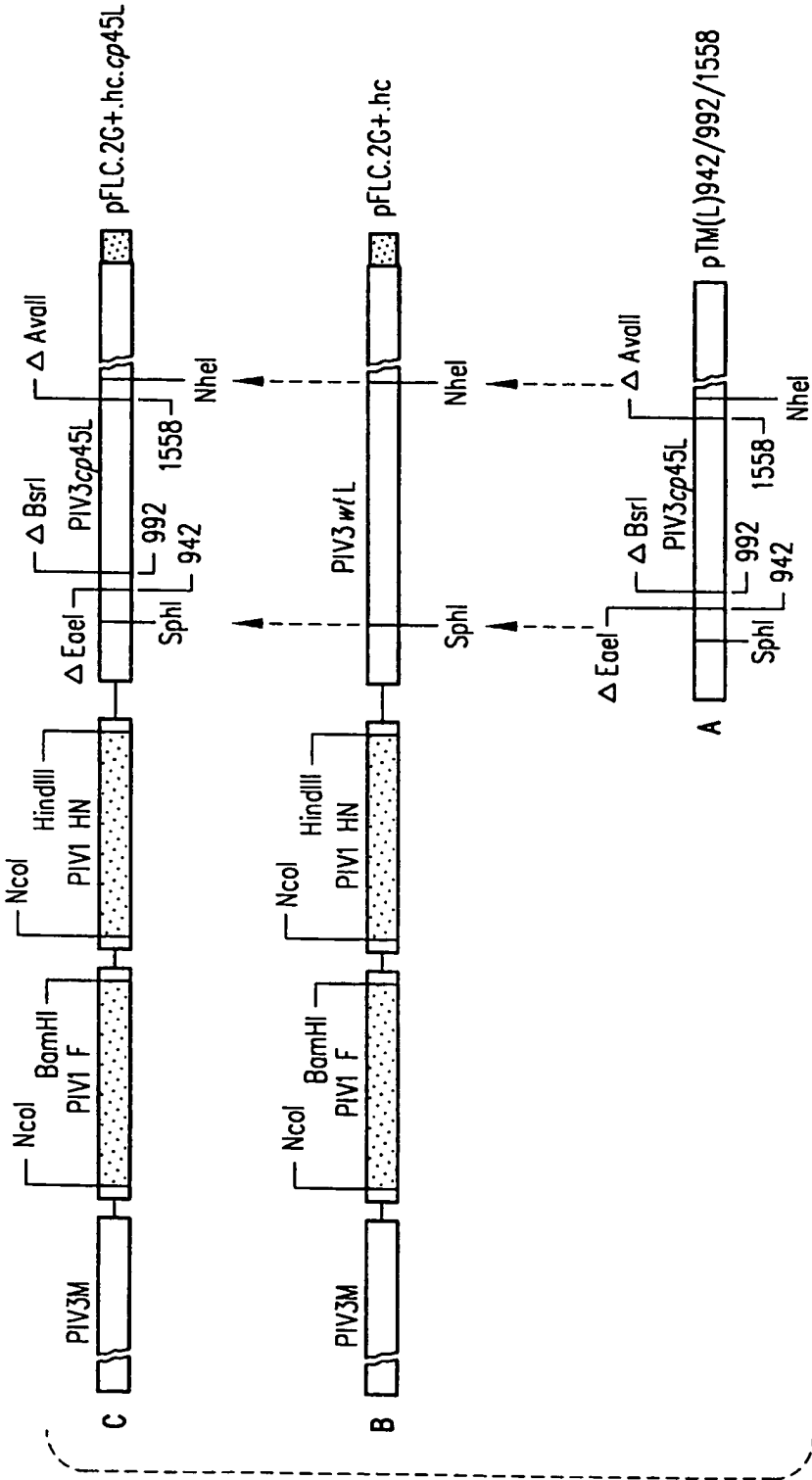
FIG. 18

FIG. 19

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6   C12N15/45   C07K14/115   C12N5/10   C12N7/01   A61K39/155

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   C12N   C07K   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 97 20468 A (UNIV ST LOUIS ;BELSHE ROBERT B (US); RAY RANJIT (US)) 12 June 1997 see abstract see page 25, line 24 – page 27, line 27 see claims 1-20 --- -/--	1-17,26, 27,30, 31, 33-69, 71-116, 118-128

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

' Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 September 1998	13/10/1998

Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Galli, I

Form PCT/ISA/210 (second sheet) (July 1992)

3

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	DIMOCK K. & COLLINS P.L.: "Rescue of synthetic analogs of genomic RNA and replicative intermediate RNA of human parainfluenza virus type 3" J. VIROL., vol. 67, no. 5, May 1993, pages 2772-2778, XP002078295 cited in the application see figure 1 ---	11,47, 52,53, 55-57, 59-61, 90,91, 93,95, 96,115
Y	EP 0 702 085 A (AKZO NOBEL NV) 20 March 1996 see the whole document ---	10-17, 22-27, 30,31, 48-72, 88, 91-98, 102-106, 117-120, 127,128
Y	STOKES A ET AL: "THE COMPLETE NUCLEOTIDE SEQUENCE OF TWO COLD-ADAPTED, TEMPERATURE-SENSITIVE ATTENUATED MUTANT VACCINE CIRUSES (CP12 AND CP45) DERIVED FROM THE JS STRAIN OF HUMAN PARAINFLUENZA VIRUS TYPE 3 (PIV3)" VIRUS RESEARCH, vol. 30, 1993, pages 43-52, XP002051711 cited in the application see the whole document	10-17, 22-27, 30,31, 48-72, 88, 91-98, 102-106, 117-120, 127,128
A	 & DATABASE GENBANK Accession No. U51116, 6 March 1997 STOKES A.: "Human parainfluenza virus 3" see the whole document ---	18-21, 28,29,32
A	STOKES A. ET AL.: "The complete nucleotide sequence of the JS strain of human parainfluenza virus type 3: comparison with the Wash/47885/57 prototype strain" VIRUS RESEARCH, vol. 25, 1992, pages 91-103, XP002078296 see the whole document & DATABASE GENBANK Accession No. Z11575, 23 August 1993 STOKES A.: "Human parainfluenza virus type 3 (wt)" see page P ---	1-128

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	COLLINS P L ET AL: "PRODUCTION OF INFECTIOUS HUMAN RESPIRATORY SYNCYTIAL VIRUS FROM CLONED CDNA CONFIRMS AN ESSENTIAL ROLE FOR THE TRANSCRIPTION ELONGATION FACTOR FROM THE 5' PROXIMAL OPEN READING FRAME OF THE M2 MRNA IN GENE EXPRESSION AND PROVIDES A CAPABILITY FOR VACCINE DEVELOPMENT" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 92, December 1995, pages 11563-11567, XP002066592 see figure 1	1,6,7, 11,43, 44,52, 84,85, 91,94, 101,116, 118,121
A	EP 0 440 219 A (SCHWEIZ SERUM & IMPFINST) 7 August 1991 see abstract	8,9,11, 45,46, 52,86, 87,91, 94,101, 118,121
A	WO 97 11093 A (CONNAUGHT LAB ;CATES GEORGE A (CA); EWASYSHYN MARY E (CA); FAHIM R) 27 March 1997 see abstract	1-11,30, 33-62, 73-101, 107-114, 118-126
P,X	TAO T. ET AL.: "Recovery of a fully viable chimeric human parainfluenza virus (PIV) type 3 in which the hemagglutinin-Neuraminidase and Fusion glycoproteins have been replaced by those of PIV type 1" J. VIROL., vol. 72, no. 4, April 1998, pages 2955-2961, XP002078294 see the whole document	1-17, 22-27, 30-128
P,X	SKIADOPOULOS M.H. ET AL.: "Three amino acid substitutions in the L protein of the human parainfluenzavirus type 3 cp45 live attenuated vaccine candidate contribute to its temperature-sensitive and attenuation phenotypes." J. VIROLOGY, vol. 72, no. 3, March 1998, pages 1762-1768, XP002078298 see the whole document	10-17, 48-69, 71,72, 88, 91-93, 95-98, 102-106, 117

-/--

3

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category°	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	DURBIN A.P. ET AL.: "Recovery of infectious human parainfluenza virus type 3 from cDNA" VIROLOGY, vol. 235, 1 September 1997, pages 323-332, XP002078299 see the whole document -----	10, 52-61, 88,89, 91-93, 96,98,99

3

Patent document cited in search report		Publication date	Patent family member(s)		Publication date	
WO 9720468	A	12-06-1997	AU	1149597 A	27-06-1997	
EP 0702085	A	20-03-1996	CA	2154023 A	19-01-1996	
			CN	1121959 A	08-05-1996	
			JP	8168381 A	02-07-1996	
EP 0440219	A	07-08-1991	AU	7007491 A	08-08-1991	
			CA	2035386 A	03-08-1991	
			JP	4211377 A	03-08-1992	
WO 9711093	A	27-03-1997	AU	6982096 A	09-04-1997	
			EP	0851873 A	08-07-1998	

(54) Title: RHABDOVIRUSES WITH REENGINEERED COATS

(57) Abstract

Replication–competent recombinant rhabdoviruses that lack a functional glycoprotein gene and express at least one foreign polypeptide such as a celluar receptor for another virus in their viral envelopes are useful in the treatment of pathogenic viruses. In one embodiment, a recombinant vesicular stomatitis virus (VSV) lacking its glycoprotein (G) gene and expressing instead the HIV receptor and a coreceptor is employed in a method for treating persons infected with HIV. The recombinant virus is defective for entry into normal cells but is able to control HIV infection in a T cell line by replicating in, and killing, HIV–infected cells.

RHABDOVIRUSES WITH REENGINEERED COATS

Related Application Data

This application claims priority benefit of co-pending U.S. application serial number 60/052,366, filed 11 July 1997.

Technical Field of the Invention

5   This invention relates primarily to replication-competent rhabdoviruses which lack a functional glycoprotein gene and express in the viral coat at least one receptor for another virus. Recombinant rhabdoviruses of the invention such as vesicular stomatitis virus which express at least one cellular receptor and a coreceptor of another pathogenic virus such as HIV are useful for the treatment of

10   patients infected with the pathogenic virus.

Background of the Invention

To cause infection, membrane-enveloped viruses such as human immunodeficiency virus type 1 (HIV-1) must first bind receptors at the cell surface and subsequently fuse their own membrane with that of the cell. This fusion process

15   releases the viral genetic material into the cytoplasm and initiates infection. Entry of HIV-1 into cells has long been known to require virus binding to the cell surface molecule CD4 (Maddon, et al., 1986; Sattentau and Weiss, 1988), and recent studies have defined chemokine receptor molecules, CXCR4 (Feng, et al., 1996; Berson, et al., 1996), CCR5 (Alkhatib, et al., 1996; Choe, et al., 1996;

Deng, *et al.*, 1996; Doranz, *et al.*, 1996; Dragic, *et al.*, 1996), and CCR3
(Choe, *et al.*, 1996; Doranz, *et al.*, 1996) as co-receptors required for entry.
HIV entry occurs in stages: initial binding of the viral envelope protein (designat-
ed gp120/41) to CD4 is followed by conformational changes that allow binding to

5   coreceptor and subsequent coalescence of the viral and cellular membranes
(Lapham, *et al.*, 1996; Trkola, *et al.*, 1996; Wu, *et al.*, 1996). HIV-infected
cells express gp120/41 on the cell surface during infection and prior to its incorpo-
ration into budding HIV-1 particles.

Recent studies have shown that vescisular stomatitis virus (VSV) has

10   potential as a high level expression vector capable of incorporating foreign
proteins into the viral envelope (Schnell, *et al.*, 1996a; Schnell, *et al.*, 1996b).
VSV causes an extremely rapid cytopathic infection in most animal cells including
human T cells in culture, but is normally non-pathogenic in humans (for review
see Wagner and Rose, 1996). Within two to three hours after infection, VSV

15   blocks host cell protein synthesis and within eight hours produces very large
quantities of progeny virus particles that bud from the cell surface prior to cell
lysis. VSV has a non-segmented, negative-strand RNA genome that is copied in
the cytoplasm of infected cells by the viral RNA polymerase to generate five
mRNAs encoding the five structural proteins. Only one of these proteins, a

20   glycoprotein designated G, is present in the viral membrane and is responsible for
the very broad host range of VSV. G protein recognizes cell surfaces and
catalyzes fusion of the viral membrane with cellular membranes (Florkiewicz and
Rose, 1984). Foreign membrane proteins such as CD4 and other viral membrane
proteins can be expressed at very high levels from the genome of recombinant

25   VSVs and these molecules are then incorporated at high levels into the viral
membrane along with G protein (Schnell, *et al.*, 1996b).

Summary of the Invention

It is an objective of the invention to utilize this strategy to engineer recombinant viruses that can be employed to target cells infected with another virus such as HIV.

5   It is a further and more specific objective to provide a method for treating a person infected with HIV.

These and other objectives are accomplished by the present invention, which provides replication-competent recombinant rhabdoviruses such as vesicular stomatitis viruses (VSV) that lack a functional glycoprotein gene and express in

10   their viral coats at least one foreign polypeptide. In preferred embodiments, the foreign polypeptide is a cellular receptor for another virus and is expressed in a manner sufficient to target cells infected with the other virus. In one embodiment, the invention provides replication-competent recombinant vesicular stomatitis virus which lacks a functional G glycoprotein gene and displays at least one cellular

15   receptor for a pathogenic mammalian virus. Where the other virus is HIV, the expressed polypeptide is an HIV receptor such as CD4, an HIV coreceptor such as CXCR4, and/or mixtures of these.

The invention correspondingly provides methods for treating patients infected with a pathogenic virus by administering to the patient an effective

20   amount of a recombinant rhabdovirus of the invention such as VSV which express-es in its viral envelope at least one receptor for the pathogenic virus. As set out hereafter, treatment methods for patients infected with HIV-1 and/or HIV-2 are disclosed.

Description of the Figures

Figure 1 illustrates diagrams of recombinant VSV genomes. The order
of the genes in wild-type VSV and in the recombinant VSVs described is shown.
Each extra gene was inserted so that it contained the appropriate signals specifying
5   VSV polymerase transcription initiation and termination. The details of plasmid
constructions and derivation of the recombinant viruses are given in the Examples.
The genes are transcribed from left to right from the negative strand RNA genome
which is shown in the 3'-5' orientation.

Figure 2 shows photographs of cultures illustratingthat VSVs lacking
10 the G gene form plaques on a complementing cell line. BHK-G cells were seeded
on 6-well plates (1.5 x 10^5 cells in each well), and G protein expression was
induced by removal of tetracycline. The indicated dilutions of 100 μl the VSVΔG-
CD4 virus (10^{-2}, 10^{-3} and 10^{-4}) were added 16 h later, followed by addition of 2 ml
of DME containing 2% methylcellulose. Cells were incubated for 48 h and then
15 stained with napthalene black to visualize the small plaques. Wild-type VSV
plaques are much larger and are visible after 18h, but plaque formation by the
defective virus on this cell line was slower, probably because of the reduced level
of complementing VSV G protein expressed.

Figure 3 shows immunoelectron microscopic visualization of CD4 in the
20 envelopes of VSVΔG-CD4 and VSVΔG-CC4. Purified wild-type VSV, VSVΔG-
CD4, or VSVΔG-CC4 particles were negatively stained with phosphotungstic acid
after labeling with a monoclonal antibody directed to CD4 followed by gold-conju-
gate goat anti-mouse IgG particles. Wild-type VSV particles contained visible
spikes of VSV G protein trimers (arrows) and did not bind antibody to CD4 (wild-
25 type VSV, left panel), while VSVΔG-CD4 or VSVΔG-CC4 bound anti-CD4
antibody and did not show visible spikes on the surface.

Figure 4 illustrates analysis of HIV-1 and VSVΔG-CC4 infection by immunofluorescence microscopy. Duplicate cultures of Jurkat cells were infected with HIV-1 strain IIIB and five days later, superinfected with VSVΔG-CC4 (panel D, E, F) or propagated without superinfection (panel A, B, C). Jurkat cells that
5   were uninfected with HIV-1 were also infected with VSVΔG-CC4 (panel G, H, I). Fourteen days after the VSVΔG-CC4 infection, cells were fixed and labeled with a mouse monoclonal antibody to VSV N protein and a secondary, affinity-purified, rhodamine-conjugated anti-mouse antibody (detected in panels C, F, I) and with a human polyclonal anti-HIV immune globulin followed by affinity-
10   purified, FITC-conjugated anti-human antibodies (detected in panels B, E, H). Panels A, D, and G show Nomarski images of the same fields of cells shown stained in the two images to their right.

Figure 5 provides line graphs illustrating quantitation of HIV-1 and VSVΔG-CC4 infection in Jurkat cells. Duplicate cultures of Jurkat cells were
15   infected with HIV-1 IIIB at day 0 and either superinfected with VSVΔ-CC4 five days later (■) or propagated without superinfection (□). The percentage of HIV$^+$ cells was determined by immunofluorescence microscopy on the days indicated (Figure 5A). The percentage of HIV$^+$ cells that were also infected with VSVΔG-CC4 was determined by immunofluorescence microscopy to detect VSV
20   N protein (Figure 5B). One hundred to two hundred cells were counted from multiple random fields to determine the percentages of cells staining positive for HIV or VSV antigens.

Figure 6 provides line graphs illustrating the effect of VSVΔG-CC4 infection on production of infectious HIV-1 and on release of RT. Media from
25   Jurkat cell cultures were analyzed for infectious HIV titers and for RT activity as described in the Examples. Titers (Figure 5A) were performed in triplicate and are reported as TCID$_{50}$/ml (□, HIV infected only; ■, HIV + VSVΔG-CC4). Titers determined by this assay were consistent with titers determined by an indirect immunofluorescence assay detecting HIV antigens. RT activity (Figure

5B) is reported in total counts per minute (cpm) bound to the DEAE paper after subtraction of a backgrounds of less than 50 cpm (□, HIV infected only; ■, HIV + VSVΔG-CC4; Δ, VSVΔG-CC4 infected only). The numbers plotted are the average of duplicates that agreed within 5%. Samples were collected from

5   Jurkat cultures on the indicated days after superinfection with VSVΔG-CC4.

Detailed Description of the Invention

This invention is based upon the construction of a new virus from vesicular stomatitis virus (VSV) that is replication-competent and kills cells rapidly, but is only able to infect cells that are first infected with HIV. The virus

10   contains a deletion in the VSV envelope glycoprotein gene and expresses instead the genes for the HIV receptor CD4 and an HIV coreceptor, CXCR4. The virus kills HIV-infected cells in culture and controls HIV infection.

In the practice of this invention, replication-competent recombinant rhabdoviruses are generated which lack a functional glycoprotein gene and express

15   a foreign polypeptide in their viral envelopes. By "foreign" is meant not naturally occurring in the rhabdovirus transformed. The foreign polypeptide is one that binds to a mammalian cell such as a cellular receptor and/or coreceptor for another virus in many embodiments, and an antibody in other embodiments. The invention is particularly suited to target cells infected with membrame-enveloped

20   viruses. The invention provides a general strategy of virus targeting to infected cells in cases where receptor(s) recognized by viral envelope proteins are known, and the viral protein has membrane fusion activity. Likewise, both conventional and hybrid antibodies to pathologic cells are also known and are used to target these cells. In either case, foreign polypeptides are expressed in the rhabdovirus

25   in a manner sufficient to target cells to be killed. Therefore, as used herein, by a "recombinant rhabdovirus lacking a functional glycoprotein gene" is meant a virus

having any alteration or disruption of the glycoprotein gene, and/or expressing a poorly functional or nonfunctional glycoprotein, or combinations thereof.

In the examples that follow, recombinant VSVs are generated which lack a functional envelope glycoprotein G or corresponding gene and express instead at least one receptor or coreceptor of an HIV virus. In preferred embodiments, the G gene is deleted, but any mutation of the gene that alters the host range specificity of VSV or otherwise eliminates the function of the G protein can be employed.

A gene for a foreign polypeptide such as one that binds to mammalian cells infected with a pathogenic virus is then inserted and/or added to the genome of the recombinant rhabdovirus. As summarized above, in many typical embodiments, the added gene is a receptor recognized by a virus glycoprotein and that is present on the surface of mammalian cells. However, recombinant rhabdoviruses engineered to kill other cells, *e.g.*, cancer cells, are encompassed by the invention. In these embodiments, genes for specific antibodies and a membrane fusion protein are typically incorporated into rhabdoviruses lacking the a functional glycoprotein gene.

The invention thus provides medical or veterinary methods for treating a patient (human being or animal) infected with a pathogenic mammalian virus by administration to the patient of an effective amount of a replication-competent recombinant vesicular stomatitis virus which lacks a functional G glycoprotein gene and displays a receptor of the pathogenic virus in its coat in a manner sufficient to target cells infected with the pathogenic virus. An example wherein the pathogenic, membrane-enveloped virus is HIV and the envelope proteins added to the VSV are an HIV receptor and a specific coreceptor is given in the Examples section hereafter. In that illustration, a T cell tropic HIV is employed, but recombinant VSVs of the invention can been engineered to express coreceptors of other HIV strains. Other embodiments express a receptor and not a coreceptor,

and some recombinant VSVs of the invention express more than one HIV corecep-
tor.

In the practice of a preferred embodiment of the invention, a patient
infected with HIV is treated by administering to the patient an effective amount of
a replication-competent recombinant vesicular stomatitis virus which lacks a
functional glycoprotein G gene and expresses at least one HIV receptor such as
CD4 and at least one HIV coreceptor such as CXCR4, CCR5, CCR3, and/or
mixtures thereof. An exemplary construct is VSVΔG-CC4 described hereafter and
illustrated in Figure 1.

Administration of a recombinant rhabdovirus of the invention to a person
or other mammal can be via any local or systemic method known by skilled
workers, but is preferably systemic. Systemic administration includes intravenous,
intramuscular, or intradermal administration by sterile injections, parenteral
administration, and the like, typically in combination with a pharmaceutically
acceptable carrier and/or other adjuvant or adjunct compound that maintains
viability of the recombinant VSV virus or other rhabdovirus, or enhances its
effect. Combinations of therapies may also be employed.

The amount of recombinant rhabdovirus necessary to bring about thera-
peutic treatment is not fixed *per se*, and necessarily depends upon the severity or
the extent of disease. Administration is facilitated and, in some cases additional
therapeutic effects are provided by, the carrier. When a carrier is employed, it is
necessary that it be inert in the sense of not inactivating the recombinant VSV or
other rhabdovirus and in the sense of not bringing about any adverse effect to the
patient to which it is administered. In most situations, doses are dependent upon
the extent of the patient's disease, the age, weight, and clinical condition of the
patient to be treated, the potency of the recombinant virus, the adjuvants or
adjunct compounds (if any) employed, and the concentrations of recombinant
rhabdovirus and other ingredients which are typically put together in association

with a pharmaceutically acceptable carrier. Most preferably, administration of the recombinant rhabdoviruses of the invention reduces the diseased cells in the patient so signficantly that they are barely detectable or undectable.

It is an advantage of the invention that preferred recombinant VSVs
5   which incorporate the receptor of one virus in the coat of another kill cells infected with the other virus rapidly and thus control infection. It is a further advantage of the invention that recombinant VSVs or other rhabdoviruses of the invention are genetically engineered to specific targets, and kill only infected or pathogenic cells.

10   The following examples are presented to further illustrate and explain the present invention and should not be taken as limiting in any regard.

Examples

In the studies reported herein VSV recombinants are generated which exhibit a complete deletion of the VSV G gene and a substitution instead of the
15   genes for CD4 and CXCR4. These viruses assemble particles containing both CD4 and CXCR4 in their envelopes and are targeted specifically to infect HIV-1-infected cells.

Experimental Procedures

Plasmid construction. Digestion with MluI and SphI was used to delete
20   the entire VSV G gene from pVSV-CD4 (Schnell et al., 1996b). The resulting plasmid was designated pVSVΔG-CD4. A plasmid encoding the CXCR4 protein with an HA epitope tag was described previously (Berson et al., 1996). CXCR4 was amplified by PCR from this plasmid with VENT polymerase (Stratagene) using primers 5'-ACTGCCCGGGCTCGAGGTTACCATGGAGGGGATCAG-3'

(SEQ ID NO: 1) and 5'-AGCTGCGGCCGCTAGCTTAGCTCCCGGGAAGAGA-
TGCG-3' (SEQ ID NO: 2). The first primer contains an _Xho_I site and the second
an _Nhe_I site (boldface letters). The PCR product was digested with _Xho_I and _Nhe_I
and cloned into pVSV-XN1 (Schnell et al., 1996a) that was digested with _Xho_I and

5   _Nhe_I. The resulting plasmid was designated pVSV-CXCR4. The G gene was
deleted from pVSV-CXCR4 with _Xho_I and _Mlu_I. The coding sequence for human
CD4 was amplified from pCD4 (Shaw et al., 1989) by PCR using primers 5'-
CCGGGTACCACGCGTACAATGAACCGGGGAGTCCCTTTTAG-3' (SEQ ID
NO: 3) and 5'-GGGCCCCTCGAGCGTGA<u>TATCTGTTAGTTTTTTT</u>CATACTC-

10   AAATGGGGCTACATGTCTTC-3' (SEQ ID NO: 4). The first primer contains
an _Mlu_I site (boldface letters); the second primer contains an _Xho_I site (boldface
letters) and a VSV transcription stop-start signal (underlined letters). The CD4
gene was inserted at the site of the G deletion to generate a plasmid that was
designated pVSVΔDG-CC4.

15   Construction of a BHK cell line expressing VSV G protein. The
tetracycline-regulated system employed was described previously (Shockett et al.,
1995). A VSV G gene was prepared from a plasmid designated pBSG by digestion
with _Xho_I. Extensions were filled in with Klenow enzyme, and the fragment
ligated to pTet-Splice (Life Technologies) that had been cleaved with _Eco_RV. The

20   resulting plasmid was called pTET-G. BHK cells on 10 cm plates (20% conflu-
ent) were transfected with 1 μg pTET-G and 10 μg pTet-TAk (Life Technologies)
using the CaPO₄ method (mammalian transfection kit; Stratagene). One day after
transfection 0.5 μg/ml tetracycline was added to suppress G protein expression,
which is toxic to BHK cells, and stable transfected cells were selected by adding

25   0.75 mg/ml geneticin (G418). Ten days after transfection, cell colonies were
isolated and, after induction by removal of tetracycline, they were screened for
expression of VSV G protein by immunofluorescence microscopy.

              Recovery of VSV recombinants. Baby hamster kidney cells (BHK-21,
ATCC) were maintained in DMEM (Dulbecco's modified Eagle's medium) supple-

mented with 5% fetal bovine serum (FBS). Cells on 10 cm dishes (~80% confluent) were infected at an MOI of 10 with vTF7-3 (Lawson et al., 1995). After 1 hour, plasmids encoding the N, P, G, and L proteins and the appropriate recombinant anti-genome RNA were transfected into the cells using a cationic

5 liposome reagent containing dimethyldioctadecyl ammonium bromide and phosphatidylethanolamine (Lawson et al., 1995; Rose et al., 1991). Plasmid amounts were 10 μg of the respective full length plasmid (VSVΔG-CD4 or VSVΔG-CC4), 3 μg pBS-N, 5 μg pBS-P, 4 μg BS-G and 2 μg pBS-L. Supernatants of recoveries were filtered through 0.22 μm filters, and 5 ml was used to infect BHK-G cells on

10 10 cm plates which had been induced for twelve hours by removal of tetracycline. Supernatants of the first passage were filtered twice through a syringe filter (0.1 μm pore size) to remove any vaccinia virus and the viruses were then passaged on 4 x 10⁶ BHK-G cells. Supernatants (10 ml) were titered on BHK-G cells and frozen at -80°C. For production of virus stocks, 2 x 10⁶ BHK cells on 10 cm

15 plates were infected for 2 h with 10 ml supernatants of VSVΔG-CD4 or VSVΔG-CC4 grown on BHK-G cells, washed three times with phosphate buffered saline (PBS) followed by addition of 10 ml RPMI medium with 10% fetal bovine serum (FBS) or 10 ml DMEM with 5% FBS. At 16 h after infection, supernatants were clarified by centrifugation and used for experiments or stored at -80˚C.

20 Preparation and analysis of viruses. For isolation of virions, a monolayer of BHK cells (~80% confluent) on a 10 cm dish was infected with VSVΔG-CD4 or VSVΔG-CC4 (multiplicity of infection, MOI=5), or wild-type VSV (MOI=0.01). At 16 h after infection, cell debris and nuclei were removed by centrifugation at 1,250 x g for 5 min and virus was then purified twice by ultra-

25 centrifugation at 120,000g through a 20% sucrose solution containing 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA. Virus pellets were resuspended and analyzed by SDS-PAGE (10% acrylamide). Gels were either stained with Coomassie brilliant blue or transferred to nitrocellulose membranes. Membranes were blocked with 5% low fat milk powder in TBST (10 mM Tris-HCl pH 8.0, 150 mM NaCl,

30 0.05% Tween™-20) for 1 h at room temperature and then incubated with an anti-

body directed against CD4 (sheep α-CD4, AIDS Research Reference and Reagent Program) or an antibody 12CA5 (Boehringer-Mannheim) directed against the HA epitope in CXCR4. Proteins were visualized after incubation with horseradish peroxidase-conjugated donkey anti-sheep IgG or horseradish-peroxidase conjugated

5   goat anti-mouse IgG (Jackson Reasearch) for 1 h at room temperature using enhanced chemiluminescence (ECL; Amersham) system.

Electron microscopy and labeling with gold-conjugated antibodies.

Virus particles from infection of approximately 10^7 cells on a 15 cm dish were recovered from the culture medium by first pelleting cell debris at 1,500 x g for

10   10 min. The virus was then concentrated and purified by centrifugation as described above. Virus samples were absorbed onto carbon coated grids for 5 min and then blocked with 1% BSA in PBS for 10 min at room temperature. The grids were then placed on a 50 μl drop of anti-CD4 Mab OKT4 (Reinherz et al., 1979) diluted 1:50 in PBS containing 1% BSA. After 1 hour, excess antibody was

15   removed by placing grids sequentially onto five 50-μl drops of 1% BSA in PBS for 2 min each time. The grids were then placed on a 50-μl drop of goat anti-mouse IgG (Fc) labeled with 15 nm gold particles (AuroProbe, Amersham Inc.). Unbound gold conjugates were removed by five sequential 2-min washes with PBS. The virus-immunogold complexes were then negative stained by incubation

20   the grids for 4 min on 50-μl drops of 2% phosphotungstic acid (ph 7.2). Excess stain was removed and the grids were air dried. Images of viruses were obtained with a Zeiss EM910 electron microscope.

Infection and immunofluorescence microscopy of infected Jurkat cells.

Approximately 3 x 10^6 Jurkat Cells (clone E6-1, ATCC #TIB-125) were seeded in

25   T-25 flasks and grown in RPMI medium supplemented with 10% FBS. Infection with HIV-1 strain IIIB was at an MOI of approximately 0.01. At five days post-infection, cells were superinfected with 5 ml of medium containing VSVΔG-CC4. As controls, one HIV-infected culture was not superinfected, and a third culture was infected only with VSVΔG-CC4. Cells were collected for analysis at each

time point as follows. One half of the volume of the culture was collected and an equal volume of RPMI supplemented with 10% FBS was added back to the original cultures. Cells were pelleted at 1000 rpm for five minutes at 5°C in an IEC Centra-7R refridgerated centrifuge (Fisher Scientific). The media was

5   discarded, cells were washed once in 5 ml PBS, and pelleted again. The PBS was removed and cells were resuspended in 100 μl PBS. The cells were then fixed in 1 ml of 3% paraformaldehyde for one hour at room temperature, or overnight at 4°C. After permeabilization in 1% Triton X-100, cells were stained with a monoclonal antibody to VSV N protein and with human polyclonal HIV im-

10   munoglobulin (AIDS Research Reference and Reagent Program), followed by rhodamine-conjugated anti-mouse and FITC-conjugated anti-human antibodies (Jackson Research).

Cells were examined and photographed with 40X planapochromat objective on a Nikon Microphot-FX microscope equipped for epifluorescence.

15   Infection of cells with HIV-1 or VSVΔG-CC4 was monitored by counting at least three random fields of cells that typically contained 100-200 cells. Syncytia were counted as single cells.

Determination of HIV Titers. HIV titers were obtained using superna-tants from infected cultures and making serial three-fold dilutions in 96-well

20   plates. Ten thousand MT-2 cells provided by the AIDS Research Reference and Reagent Program (ARRRP) were added to each well and the virus and cells were mixed by pipeting. Plates were incubated at 37°C for 3-5 days and scored for the presence of syncytia. Titers at each time point were performed in triplicate and two or more syncytia per well were counted as positive for HIV infection.

25   Titers were calculated as described previously (Reed and Muench, 1938) and are reported as 50% tissue culture infectious doses($TCID_{50}$) per ml.

Reverse Transcriptase Assays. Each assay was performed in 96-well plates in duplicate (Willey et al., 1988). Twenty-five microliters of reverse

- 14 -

transcription reaction mix (0.05% NP-40, 50 mM Tris-HCl, pH 7.8, 75 mM KCl,
2 mM DTT, 5 mM MgCl$_2$, 5 units oligo (dT)12-18, 5 mg/ml poly A) was added,
followed by addition of 10 μl of cell-free culture supernatants. An additional 25
μl of reaction mix was added containing 10 μCi/ml α-[^{32}P]-dTTP, and the reaction

5   was placed at 37°C for 90 minutes. Five microliters of each sample was then
spotted onto Whatman DE81 paper, which was allowed to air dry. The DE81
paper was then washed in 2 x SSC (0.3 M NaCl, 20 mM sodium citrate) buffer
four times, each time at room temperature for five minutes, followed by two one-
minute washes in 95% ethanol at room temperature. After air drying, the paper

10   was exposed to a Molecular Dynamics PhosporImager screen overnight. Samples
were quantitated directly on the phosphorimager and by counting in a scintillation
spectrometer after excision from the paper. Duplicate samples agreed within 5%.

       Neutralization of infection by VSVDG-CC4. Polyclonal sheep anti-CD4
serum and anti-gp120 serum (ARRRP) were heat treated at 56°C for one hour to

15   inactivate complement. The anti-VSV neutralizing monoclonal antibody (I1) was a
mouse ascites fluid. Neutralizations were performed as follows. VSVΔG-CC4
virus (1 ml derived from ~ 10^6 infected BHK cells) was mixed with 100 μl anti-
CD4 or 100 μl anti-VSV for one hour at 37°C, and then the mixture was added to
~ 10^6 Jurkat cells that had been infected with an MOI of 0.01 HIV-1 IIIB five

20   days previously. Alternatively, 100 μl anti-gp120 serum was added directly to 1
ml of HIV-infected Jurkat cells for one hour at 37°C. Then 1 ml of VSVΔG-CC4
was added to the cells. After 3 days, cells were pelleted at 1000 rpm in a clinical
centrifuge for 5 minutes and washed once in PBS. Cells were fixed and stained to
detect HIV and VSV N protein antigens.

25   Results

       Recovery of VSV recombinants expressing CD4 and CXCR4. Genetic
engineering of non-segmented negative-strand RNA viruses such as VSV is
complicated by the fact that the minimal infectious unit of the virus is not RNA,

but rather the viral RNA genome in a ribonucleoprotein complex composed of
nucleocapsid (N) and polymerase proteins (L and P). Infectious VSV can be
recovered from plasmid DNA as follows. Four separate plasmids expressing the
full-length antigenomic VSV RNA and the N, P and L genes under control of

5   bacteriophage T7 promoters are transfected into cells already infected with a
vaccinia virus recombinant expressing T7 RNA polymerase (vTF7-3). Intracellu-
lar assembly of the antigenomic RNA into nucleocapsids is followed by VSV
replication and transcription to generate infectious, replication-competent VSV
(Fuerst et al., 1986; Lawson et al., 1995; Whelan et al., 1995). Additional genes

10 can be expressed from extra transcription units introduced into the VSV genome
(Schnell et al., 1996a).

The construction of a VSV recombinant expressing the CD4 protein in
addition to the five VSV proteins N, P, M, G and L has been previously reported
(Schnell et al., 1996b). To generate a recombinant expressing CD4 and CXCR4

15 instead of VSV G, the upstream VSV G gene was deleted from the VSVCD4 con-
struct (Figure 1). The recovery of virus, designated VSVΔG-CD4, was then
accomplished by including a complementing plasmid encoding VSV G protein in
addition to those encoding N, P, and L. The gene encoding CXCR4 at the
position downstream of CD4 was next added and the virus designated VSVΔG-

20 CC4 was recovered. Because the recovered viruses are capable of only a single
round of infection on BHK cells, they were initially recovered and grown on cells
infected with a vaccinia virus encoding the T7 RNA polymerase and transiently
expressing a complementing VSV G protein from a transfected plasmid DNA.

A cell line expressing VSV G protein allows growth of the defective

25 viruses. In order to grow the VSVΔG-CD4 and VSVΔG-CC4 virus in the absence
of vaccinia virus, a BHK cell line expressing the VSV G protein from an inducible
promoter (Shockett et al., 1995) was generated. Figure 2 shows that the VSVΔG-
CC4 virus forms plaques on this cell line indicating multiple rounds of infection.
Titers obtained after growing the defective viruses on this line were in the range of

- 16 -

0.5 - 1 x 10^6 pfu/ml. These titers are reduced at least 1000-fold compared to those of wild-type VSV presumably because the levels of G protein provided by the complementing cell line are low compared to those expressed in wild-type infection.

5   Incorporation of CD4 and CXCR4 into the recombinant viruses. To determine if the CD4 and CXCR4 molecules were expressed from the defective viruses and incorporated into virus particles, BHK cells (not expressing VSV G) were infected with VSVΔG-CD4 or VSVΔG-CC4 that had been grown on the BHK-G line (MOI = 5). After 16 hours when all cells had been killed, the virus

10   particles were purified from the medium. Viral proteins were then separated by SDS-PAGE and detected by staining with Coomassie blue. The wild-type virus contains the L, G, N, and M proteins (P is a minor protein comigrating with N) while the VSVΔG-CD4 and VSVΔG-CC4 viruses lack G but contain a new protein of about 55,000 daltons, the size expected for CD4. The presence of CD4 in the

15   particles was also verified by western blotting with sheep anti-CD4 antibody or monoclonal antibody 12CA5 to the HA epitope tage in CXCR4. The CXCR4 protein was not detected in VSVΔG-CC4 by staining, perhaps because it contains seven membrane spanning segments that does not bind the stain, but was easily detected by western blotting.

20   Budding of VSV in the absence of VSV G protein is known to be approximately 20-fold less efficient than budding in its presence (Knipe et al., 1977). Similar results have been obtained for rabies virus (Mebatsion et al., 1996). Although the amounts of protein detected by Coomassie blue staining appeared similar for all viruses, the virus loaded in test lanes were derived from 10^7

25   infected BHK cells, while the wild-type virus in the control lane was from only 2 x 10^5, or 50-fold fewer infected cells to achieve similar staining. The results not only confirm the importance of VSV G protein in driving highly efficient virus budding, but also illustrate that significant amounts of virus production occur even in the absence of VSV G protein.

To examine the morphology of the recombinant virus particles and to
verify that CD4 was exposed on the viral membrane, purified virus particles were
examined by electron microscopy after labeling with antibody to CD4 and a gold-
conjugated secondary antibody. Photographs of negatively stained virus particles

5   are shown in Figure 3. Wild-type VSV particles contained visible G protein
spikes (arrows) and did not bind antibody to CD4, while the VSVΔG-CD4 or
VSVΔG-CC4 viruses lacked visible spikes and did bind anti-CD4 antibodies. The
antibody to CXCR4 used in western blotting recognizes the internal C-terminal
HA tag on CXCR4 and therefore is not suitable for labeling virus particles.

10   <u>Infection of HIV-1 Infected Cells by VSVΔG-CC4.</u> Because cells
infected with HIV-1 have the gp 120/41 envelope protein on their surface, it might
be possible to infect these cells with VSVΔG-CC4. The gp 120/41 molecules
present on the cell surface should bind to CD4 and CXCR4 on the virus surface
and promote fusion of the viral and cell membranes. To test this possibility, a

15   human T cell line (Jurkat) was infected with HIV-1 IIIB. At five days post-
infection, it was expected from previous experiments that about half of the cells
would be infected with HIV-1, and VSVΔG-CC4 virus was added. Two control
cultures, one infected with HIV-1 only, and another infected with VSVΔG-CC4
only, were followed in parallel.

20   Initially infection by HIV-1 and VSVΔG-CC4 using indirect immuno-
fluorescence microscopy to detect both HIV-1 proteins and VSV nucleocapsid (N)
protein was followed. An example of results from the immunofluorescence
analysis at 19 days after HIV infection is shown in Figure 4, along with a Nomar-
ski image of the same field of cells. A compilation of the immunofluorescence

25   data obtained throughout the experiment is graphed in Figure 5. The fields shown
in Figure 4 illustrate that in the control culture infected with HIV-1 only, nearly
all cells stained positive for HIV antigens (A and B) while none of the cells stained
positive for VSV N protein (C). In the cells infected with HIV-1 and VSVΔG-
CC4, only a small fraction of the cells were positive for HIV-1 antigens (D and

- 18 -

E), and the majority of those cells were also positive for VSV N protein (F),
indicating that they had been superinfected with VSVΔG-CC4. It was also noted
that rare cells that were strongly positive for VSV N protein expression, had only
little or no HIV protein evident. These cells may result from shut-off of cellular

5   and HIV protein synthesis by VSVΔG-CC4 or from other mechanisms of infection
(see Discussion below). In Jurkat cells infected with VSVΔG-CC4 only, none of
the cells showed the presence HIV or VSV N antigens (H, I, and J).

From the quantitation in Figure 5 it is evident that when cells were
infected with HIV-1 alone, the percentage of HIV-1$^+$ cells rose to nearly 100%

10   and remained high throughout the experiment. When cells were first infected with
HIV-1 and subsequently infected with VSVΔG-CC4, the percentage of HIV$^+$ cells
began to decrease at day 14 and plateaued at day 21 to levels between 5 and 10%
HIV$^+$. In addition, Figure 5 shows that the percentage of HIV-1$_+$ cells that were
also infected with VSVΔG-CC4 increased and then reached a plateau at around 90-

15   95%. It therefore appears that cells infected with HIV-1 are rapidly superinfected
with VSVΔG-CC4 and killed before high levels of HIV-1 can be released. A
balance is then reached between the two viruses where HIV infection is controlled
by VSVΔG-CC4 infection and maintained at low levels. Very similar results were
obtained in an experiment performed with the H9 T cell line, another cell line in

20   which HIV-1 IIIB grows well.

The possibility that the VSVΔG-CD4 virus lacking the coreceptor gene
might infect HIV-1 infected cells was also tested. In one experiment it was
observed that a low level of infection by VSVΔG-CD4 (about 10% that observed
with VSVΔG-CC4) occurred. However, the initial infection could be completely

25   neutralized with antibody to VSV G, indicating that a trace of G protein
sufficient to start infection must have been present in the virions. This
G protein was presumably carried into the BHK cell line from the input virus
grown in the BHK-G line and then incorporated into exiting virions. Interestingly,
once the infection was established in HIV-infected Jurkat cells, it persisted for

several weeks, indicating that the virus could propagate without encoding its own

coreceptor. Presumably budding virions can pick up cell-encoded coreceptor and

are then able to propagate. However, control of HIV-1 infection was not as

strong as with VSVΔG-CC4, indicating that it is preferable to have the virus

5   encode high levels of the coreceptor. Because the control of HIV infection was

less effective without coreceptor, further studies with this virus were not pursued.

VSVΔG-CC4 infection controls release of infectious HIV-1. To obtain

measurements of the effects VSVΔG-CC4 on production of infectious HIV-1 and

on release of HIV-1 particles, the experiments shown in Figures 4 and 5 were

10   repeated. but instead followed HIV-1 titers and release of reverse transcriptase

(RT) into the medium (Figures 6A and 6B). At the time of addition of VSVΔG-

CC4 at three days after HIV-1 infection, HIV titers and RT levels in the medium

were low. In the control cells infected with HIV-1 alone or in the cells super-

infected with VSVΔG-CC4, both the HIV titers and RT activity peaked at ten days

15   after infection. There was then a decline in RT activity by day 14 in cells

superinfected with VSVΔG-CC4 and a leveling off at about 10% of the RT level

seen in control cells. The results with titers of infectious HIV-1 were much more

dramatic, with a complete disappearance of infectious HIV-1 (> 4 logs below the

control) at day 14 followed by a fluctuation at levels of only 0-39 infectious

20   particles per milliliter out to day 34. These titers should be compared to persistent

titers of $\sim 10^4$/ml for the control culture that was not infected with VSVΔG-CC4.

The RT results were consistent with what has been seen previously with

the immunofluorescence in Figure 5, but the much larger decreases in infectious

HIV titers were not expected. These lower titers could be explained if the

25   VSVΔG-CC4 infection selected for HIV-infected cells that expressed little or no

HIV envelope and thus produced HIV particles lacking envelope but containing

RT.

Anti-CD4 or anti-gp120 block infection by VSVΔG-CC4. Control

neutralization experiments (Table 1) to verify that entry of VSVΔG-CC4 into HIV

infected cells was dependent on CD4 and gp120 were also performed. VSVΔG-

CC4 was incubated with antibody to CD4, neutralizing antibody to VSV-G, or

5   without antibody before addition to Jurkat cells that had been infected with HIV-1

for five days as in Figure 5. Table 1 shows that anti-CD4 completely eliminated

the VSVΔG-CC4 infection, while antibody to G did not.

Table 1. Neutralization of VSVΔG-CC4 Infectivity		
Antibody[a]	% HIV[+] Cells[b]	% VSV N[+] Cells[b]
None	60	39
anti-VSV G	59	37
anti-CD4	74	0
anti-HIV gp 120	71	0

[a] Viruses grown on BHK cells not expressing VSV G were incubated with anti-
15   VSV G or anti-CD4, or cells were incubated with anti-HIV gp120 prior to mixing
of virus and cells.

[b] HIV positive and VSV N^{+} cells were identified by indirect immunofluorescence
microscopy as in Figures 4 and 5. Approximately 100 cells from random fields
were counted for each determination. The percentage of cells positive for HIV
20   antigens or VSV N protein is indicated.

Similarly, pre-incubation of the HIV-infected cells with a polyclonal anti-gp120

antibody completely prevented infection by VSVΔG-CC4. Because CD4 can bind

MHC class II on cell surfaces (Doyle and Strominger, 1987), it was conceivable

that some low level of infection could occur after binding of VSVΔG-CC4 to

25   MHC class II on cells, although this would not be expected without a membrane

fusion protein. To test this possibility Daudi cells, a lymphocyte cell line known

to express abundant quantities of MHC class II protein (Till et al., 1988), was

used. These cells were readily infected by wild-type VSV, but no infection by VSVΔG-CC4 was observed.

Discussion

A recombinant VSV that lacks its own envelope protein gene (G) and
5   expresses instead the HIV-1 receptor and coreceptor molecules, CD4 and CXCR4
was constructed. This novel virus is defective for entry into normal cells, but can
be propagated on cells expressing a complementing VSV envelope protein. The
complemented virus can then be grown on cells not expressing G protein where it
buds particles containing CD4 and CXCR4 in the viral membrane. These viruses
10   are replication competent, but can enter and propagate only in cells that are first
infected with HIV-1 and express HIV-1 envelope protein. Infection of these cells
by VSVΔG-CC4 is neutralized by antibody to CD4 added to the virus, or by
antibody to HIV gp120 added to the cells.

The results showed that in a mixed population of cells about half of
15   which were HIV-1-infected and half were uninfected, superinfection with VSVΔG-
CC4 ultimately limited production of infectious HIV-1 to levels that were barely
detectable or undetectable, at least 300-fold to 104-fold lower than HIV titers
produced by control cells that were not superinfected with VSVΔG-CC4. This
reduction presumably is the result of killing of the HIV-1 infected cells by the
20   VSVΔG-CC4 virus. The reduction in HIV-1 titers was greater than would have
been predicted from analysis of the number of HIV-1-infected cells or from RT
assays. The number of HIV positive cells was reduced about ten-fold by the
VSVΔG-CC4 infection, and the release of RT was reduced to a similar extent. In
considering the mechanism of VSVΔG-CC4 action, this discrepancy is reasonable
25   since VSVΔG-CC4 will target and kill those cells expressing HIV envelope protein
and thus select for cells that produce little or no HIV envelope. Such cells would
still score positive for HIV-1 antigen production, and would produce normal levels

of HIV-like particles containing RT, but the majority of these particles would be noninfectious.

The persistence of a low level of both HIV-1 and VSVΔG-CC4 coinfection for over one month in culture indicates that both viruses are continuing to replicate and that they have reached a balance. Based on the data, it seems unlikely that VSVΔG-CC4 will ever completely eliminate the HIV-1 infection in the culture. This low level persistence may occur because VSVΔG-CC4 can only infect HIV-1 infected cells after gp120/41 is expressed on the cell surface and such cells likely release some HIV-1 before they are killed.

The VSVΔG-CC4 virus infects cells that have already been infected with HIV-1, but it may also be fusing directly with HIV-1 virions. Such fused viruses would contain the nucleocapsids of HIV and VSV and might be able to initiate infection if sufficient gp120/41 were still present on the membrane. Such an infection would almost certainly favor replication of VSVΔG-CC4 because VSV would shut off cellular protein synthesis in 2-3 hours, even before HIV had sufficient time for reverse transcription and integration. Infection by these fused viruses could explain the presence of cells (Figure 4) that are making VSV proteins but do not appear to have synthesized HIV proteins. While not wishing to be bound to any theory, it is also possible that VSV has inhibited HIV protein synthesis in these cells. Infection of uninfected cells by VSVΔG-CC4 might also occur if VSVΔG-CC4 pseudotypes carrying HIV envelope protein were generated in HIV-infected cells. Incorporation of the wild-type HIV envelope into VSV particles is extremely inefficient (Johnson et al., 1997) and therefore this latter mechanism would likely be rare. To test directly for such pseudotypes, a short-term infection of Jurkat cells with supernatants from cells coinfected with HIV-1 and VSVΔG-CC4 was carried out. VSV protein expression was not detected in these cells, while it was readily detected by using the same supernatant to infect HIV-infected Jurkat cells. It was concluded that the VSVΔG-CC4(HIV) pseudotypes are extremely rare if they exist at all.

- 23 -

Several aspects of this system that are worth noting. First, because VSVΔG-CC4 is specifically targeted to cells already infected with HIV-1, the VSΔG-CC4 infection level would be expected to parallel HIV infection and disappear with clearance of HIV. Second, VSVΔG-CC4 encodes only human proteins as its envelope proteins, and thus would not induce production of neutralizing antibodies. The internal VSV proteins would be expected to induce cytotoxic T cell responses, but such responses might help in clearing HIV infection by rapidly killing cells infected with HIV-1 and VSVΔG-CC4. Third, mutations to drug resistance are rapidly selected in HIV-1, but resistance to infection by VSVΔG-CC4 would require loss of HIV's ability to bind CD4 or coreceptor and would therefore not be selected.

The results also illustrate an important aspect of VSV budding and assembly. Based on virus protein yields and titers, it is estimated that each BHK cell infected with wild-type VSV produces approximately 100,000 particles, and up to 10% of these particles are infectious. The deletion of the VSV G gene reduced particle yields of VSVΔG-CC4 or VSVΔG-CD4 approximately 30-50 fold, indicating a substantial role for G in promoting budding. However, despite this reduced budding, at least 2,000 particles of VSVΔG-CC4 or VSVΔG-CD4 are produced from each BHK cell in the absence of G protein. The incorporation of CD4 and CXCR4 into these particles is presumed to occur by a passive process of protein trapping as virus buds from the cell surface, because neither protein would be expected to contain specific signals promoting incorporation. Because VSV blocks host protein synthesis and expresses extremely large amounts of its mRNA and proteins, CD4 and CXCR4 are likely to be the major proteins present on cell surface and therefore the major proteins in the VSVΔG-CC4 envelope.

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The papers cited herein are expressly incorporated in their entireties by reference.

5   The above description is for the purpose of teaching the person of ordinary skill in the art how to practice the present invention, and it is not intended to detail all those obvious modifications and variations of it which will become apparent to the skilled worker upon reading the description. It is intended, however, that all such obvious modifications and variations be included within the

10   scope of the present invention, which is defined by the following claims. The claims are intended to cover the claimed components and steps in any sequence which is effective to meet the objectives there intended, unless the context specifically indicates the contrary.

- 28 -

CLAIMS

1. A replication-competent recombinant rhabdovirus which lacks a functional glycoprotein gene and encodes at least one foreign polypeptide that is expressed in its viral envelope.

2. A recombinant virus according to claim 1 wherein the polypeptide is a cellular receptor of another virus which is expressed in the rhabdovirus in a manner sufficient to target cells infected with the other virus.

3. A recombinant virus according to claim 1 which is a vesicular stomatitis virus which lacks a functional G glycoprotein gene and encodes at least one cellular receptor of a pathogenic mammalian virus.

4. A recombinant virus according to claim 3 wherein the pathogenic virus is HIV-1 or HIV-2.

5. A recombinant virus according to claim 4 wherein the pathogenic virus is HIV-1.

6. A recombinant virus according to claim 4 which expresses in its viral envelope at least one HIV receptor, at least one HIV co-receptor, or mixtures thereof.

7. A recombinant virus according to claim 3 comprising the additional genomes illustrated in Figure 1.

8. A method for treating a patient infected with a pathogenic virus comprising administering to the patient an effective amount of a recombinant virus according to claims 1, 2, 3, 4, 5, 6, or 7 which expresses in its viral envelope at least one receptor of the pathogenic virus so that the patient's cells harboring the pathogenic virus are targeted.

5

9. A replication-competent recombinant vesicular stomatitis virus which lacks a functional glycoprotein G gene and expresses at least one HIV receptor and at least one HIV co-receptor.

10. A method for treating a patient infected with HIV-1 or HIV-2 by administering to the patient an effective amount of a recombinant virus according to claims 1, 2, 3, 4, 5, 6, 7, or 9.

Figure 1
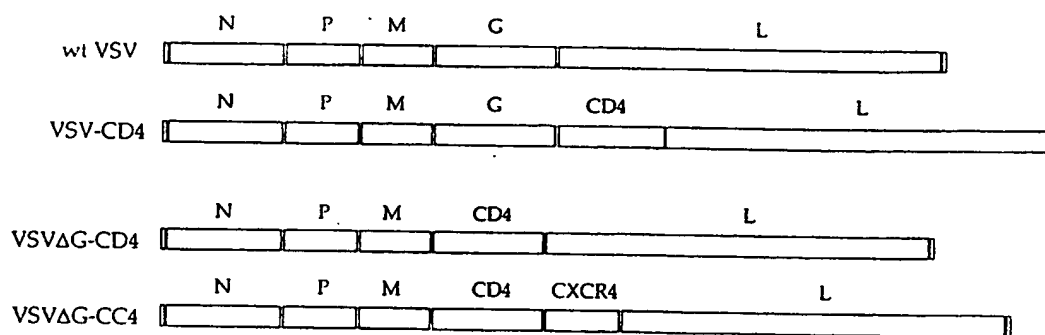
Figure 2
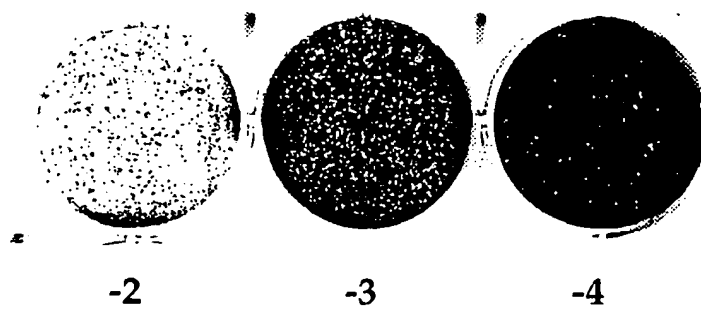
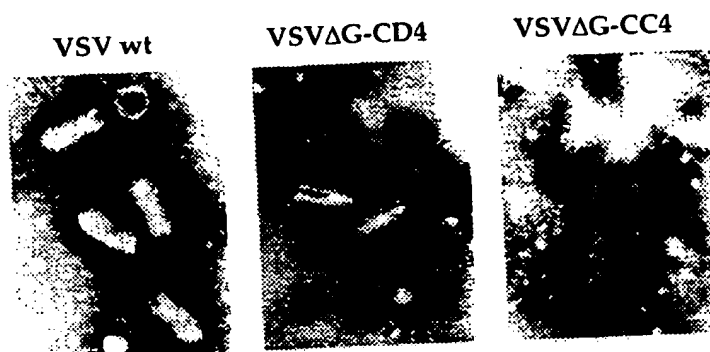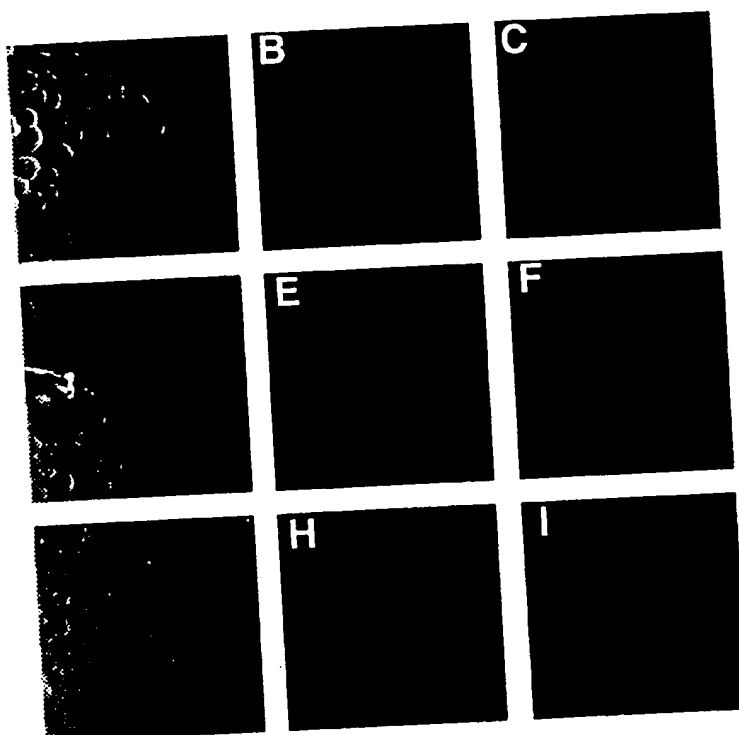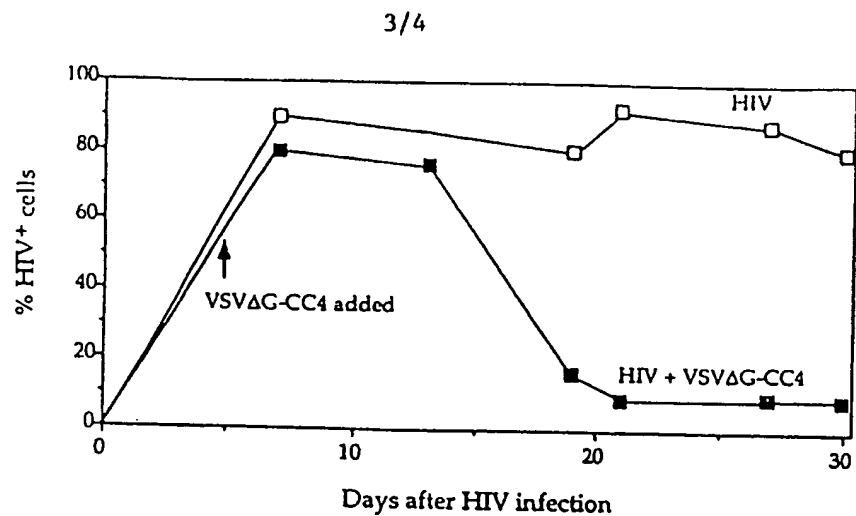VSV wt   VSVΔG-CD4   VSVΔG-CC4

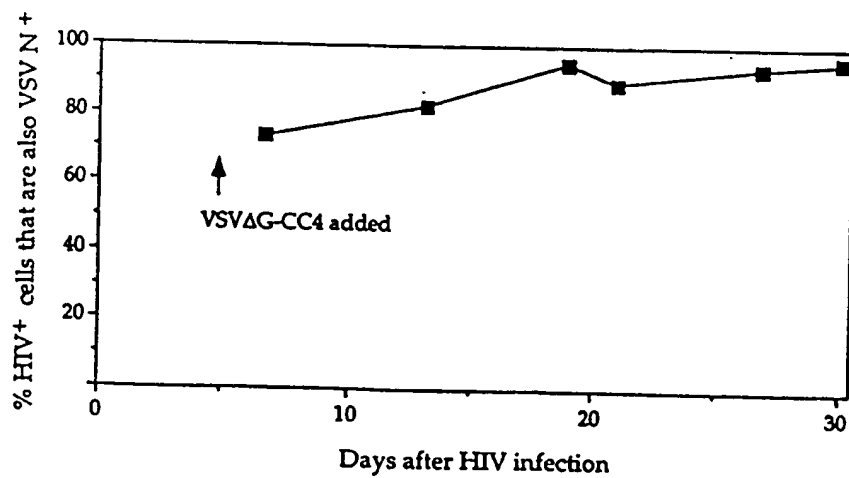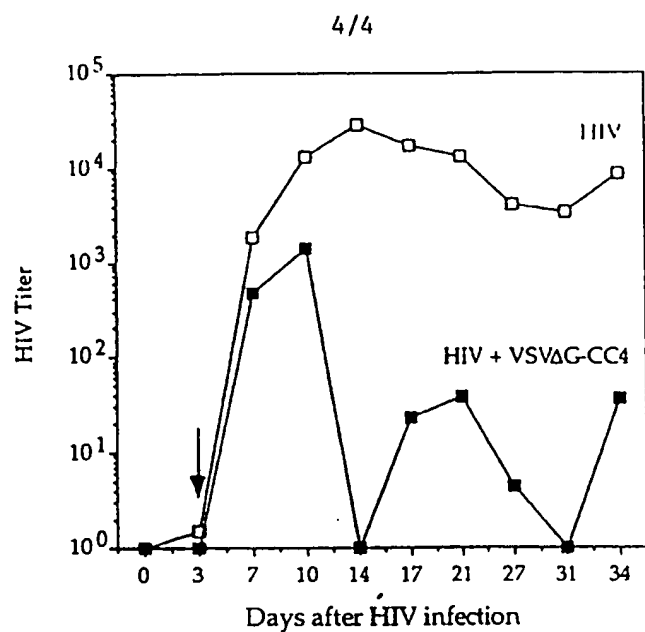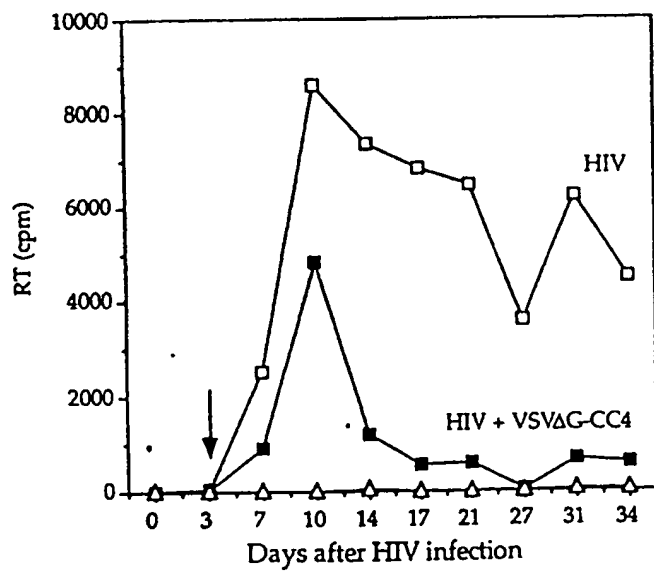**Figure 3**

Figure 4

Figure 5A

Figure 5B

4/4

Figure 6A

Figure 6B

1/3

SEQUENCE LISTING

(1) GENERAL INFORMATION:

   (i) APPLICANTS: John K. Rose
                     Matthias Schnell
                 Erik Johnson

   (ii) TITLE OF INVENTION: Rhabdoviruses with Reen-
gineered Coats

      (iii) NUMBER OF SEQUENCES: 4

      (iv) CORRESPONDENCE ADDRESS:

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      (v) COMPUTER READABLE FORM:

           (A) MEDIUM TYPE: 3.5" 1.44 Mb diskette
           (B) COMPUTER: IBM PC
           (C) OPERATING SYSTEM: MS DOS
           (D) SOFTWARE: Word Processing

      (vi) CURRENT APPLICATION DATA:

           (A) APPLICATION NUMBER: PCT/US/98/
           (B) FILING DATE: 10-JUL-1998
           (D) CLASSIFICATION:

      (vii) PRIOR APPLICATION DATA:

           (A) APPLICATION NUMBER: 60/052,366
           (B) FILING DATE: 11-JUL-1997

      (viii) ATTORNEY INFORMATION:

           (A) NAME: Mary M. Krinsky
           (B) REGISTRATION NO.: 32423
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      (ix) TELECOMMUNICATION INFORMATION:

           (A) TELEPHONE NUMBER: 203-773-9544
           (B) TELEFAX NUMBER: 203-772-0587

2/3

(2) INFORMATION FOR SEQ ID NO: 1:

    (i)   SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 36
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE:
       (A) DESCRIPTION: DNA

    (ix)   FEATURE:
       (A) NAME/KEY: primer used in constructs

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ACTGCCCGGG CTCGAGGTTA CCATGGAGGG GATCAG   36

(3) INFORMATION FOR SEQ ID NO: 2:

    (i)   SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 38
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE:
       (A) DESCRIPTION: DNA

    (ix)   FEATURE:
       (A) NAME/KEY: primer used in constructs

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO: 2:

AGCTGCGGCC GCTAGCTTAG CTCCCGGGAA GAGATGCG   38

(4) INFORMATION FOR SEQ ID NO: 3:

    (i)   SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 41
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE:
       (A) DESCRIPTION: DNA

    (ix)   FEATURE:
       (A) NAME/KEY: primer used in constructs

3/3

(xi)   SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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(5) INFORMATION FOR SEQ ID NO: 4:

        (i)   SEQUENCE CHARACTERISTICS:
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                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE:
                (A) DESCRIPTION: DNA

        (ix)   FEATURE:
                (A) NAME/KEY: primer used in constructs

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GGGCCCCTCG AGCGTGATAT CTGTTAGTTT TTTTCATACT   40

CAAATGGGGC TACATGTCTT C   61

International application No.

PCT/US98/14527

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(6)   : C12N 7/00, 7/04, 15/00; A61K 39/21, 39/205
US CL   : 435/235.1, 236, 320.1; 424/199.1, 224.1
According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   435/235.1, 236, 320.1; 424/199.1, 224.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

USPATFUL, WPIDS, MEDLINE

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LAWSON, N.D. et al., Recombinant vesicular stomatitis viruses from DNA. Proc. Natl. Acad. Sci. USA. May 1995, Vol. 92, pages 4477-4481, especially page 4478.	1-7 and 9
Y	MEBATSION, T. et al., Highly stable expression of a foreign gene from rabies virus vectors. Proc. Natl. Acad. Sci. USA. July 1996, Vol. 93, pages 7310-7314, especially page 7311.	1-7 and 9
Y	SCHNELL, M. J. et al., Foreign glycoproteins expressed from recombinant vesicular stomatitis viruses are incorporated efficiently into virus particles. Proc. Natl. Acad. Sci. USA. October 1996, Vol. 93, pages 11359-11365, especially 11360.	1-7 and 9

[X]	Further documents are listed in the continuation of Box C.	[ ]	See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
		"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 AUGUST 1998	**17 SEP 1998**

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer *[signature]* Jeffrey S. Parkin, Ph.D.
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FENG, Y. et al., HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane G protein-coupled receptor. Science. 10 May 1996, Vol. 272, pages 872-877, see entire document.	6